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Chemotaxis

Methods and Protocols

Edited by

Tian Jin

Dale Hereld

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Edited by

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Preface

Cell movement is fundamental to the development and other vital functions of organisms. Many motile cells can detect shallow gradients of specific chemical signals in their environments and migrate accordingly. This directed cell movement is called chemotaxis and is essential for various cell types to carry out their biological functions. This book includes state-of-the-art methods for investigating cell migration behaviors, studying molecular components involved in detecting extracellular signals and directing cell movement, visualizing spatiotemporal dynamics of the components in signaling networks of chemotaxis in real time, and constructing quantitative models that simulate chemoattractant-induced cell responses.

Various methods to investigate cell movement are presented in Chapters 1–16. These chapters contain experimental procedures to visualize and measure migration behaviors of different kinds of organisms, including bacterial movement in chemoattractant gradients, light-induced responses of prokaryotes, *Chlamydomonas* and *Dictyostelium discoideum*, electric field-directed movement of eukaryotic cells, chemotropism in the budding yeast, cell migration of *D. discoideum*, *C. elegans*, *Drosophila*, zebrafish, and mouse, chemotaxis of *D. discoideum*, and neutrophil-like cell lines. The volume also contains microscopy procedures to study breast cancer cell migration, tumor cell invasion in vivo, and neuronal chemotaxis. These methods provide quantitative measurements and description of cell migration behaviors.

Significant progress has been made in recent years toward identifying the molecular components and understanding the molecular networks that underlie chemoattractant sensing and cell migration in various organisms. Chapters 17–20 describe the methods to study signal transduction pathways involved in chemotaxis in the model system, *D. discoideum*. Chapter 21 introduces the role of chemokine receptor signaling in HIV infection.

Fluorescence microscopy permits us to directly monitor dynamics of many signaling events in single cells in real time. Chapters 22–29 describe methods that measure spatiotemporal dynamics of chemoattractant concentrations, activation of receptors, heterotrimeric G-proteins, small G-protein Ras signaling, and actin cytoskeleton assembly using different imaging techniques. Several chapters introduce cutting-edge imaging techniques, such as FRAP, FRET, and single-molecule microscopy, to determine mobility of receptors and other signaling components. These techniques allow us to reveal dynamics of signaling components in live cells and to track signaling events in single cells in space and time.

Computer-based quantitative models that address the complexity of a signaling network with its many interacting components are valuable for studies of chemotaxis. Chapter 30 summarizes a computer program that quantifies movement of amoeboid cells. Chapter 31 introduces mathematical calculations on experimentally generated chemoattractant gradients. Finally, Chapters 32 and 33 introduce two computational models that are constructed to simulate spatial–temporal dynamics of signaling networks for eukaryotic chemosensing.

We are grateful to all the authors for contributing their expertise and believe that this book will provide the reader with an overview of and practical guidance on the diverse methodologies that are propelling chemotaxis research forward.

Rockville, MD
Rockville, MD

Tian Jin
Dale Hereld

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Chapter 1

Microfluidic Techniques for the Analysis of Bacterial Chemotaxis

Derek L. Englert, Arul Jayaraman, and Michael D. Manson

Summary

Anton van Leeuwenhoek first observed bacterial motility in the seventeenth century, and Wilhelm Pfeffer described bacterial chemotaxis in the late nineteenth century. A number of methods, briefly summarized here, have been developed over the years to quantify the motility and chemotaxis of bacteria, but none of them is totally satisfactory. In this chapter, we describe two new assays for chemotaxis that are based on microfabrication and microfluidic techniques. With easily culturable and manipulated bacteria like *Escherichia coli*, fluorescent labeling of the cells with green fluorescent protein (GFP) or red fluorescent protein (RFP) provides a convenient method for visualizing cells and differentiating two strains in the same experiment. The methods can be extended to environmental samples and mixed bacterial populations with suitable modifications of the optical recording system. The methods are equally useful for studying random motility, attractant chemotaxis, or repellent chemotaxis. The microfluidic system also provides a straightforward way to enrich for mutants that lose or gain responses to individual chemicals. The same approaches can presumably be used to isolate bacteria from environmental samples that respond, or do not respond, to particular chemicals or mixtures of chemicals.

Key words: Bacterial motility, Bacterial chemotaxis, Behavioral assays, Miniaturized plug-in-pond assay, Microfluidic chambers, Enrichment for mutants or natural isolates

1. Introduction

1.1. Brief Review of Bacterial Motility and Chemotaxis

Anton van Leeuwenhoek made the first observations of bacterial motility at the dawn of microbiology. In 1676, he lovingly described the movements of the little animalcules he saw with his homemade microscopes. In the late nineteenth century, Wilhelm Pfeffer in Tuebingen described how bacteria are attracted to nutrients. The subject lay largely dormant again for another three-quarters of a century until the mid-1960s, when Julius Adler began to systematically describe the chemotactic behavior of *Escherichia coli*.

Adler's earliest published work on chemotaxis (1) caught the attention of many very talented individuals. His meticulous description of how bacteria perceive molecules without importing or metabolizing them (2) established that there must be specific cell-surface receptors that modulate the swimming behavior of the cells. These discoveries attracted other outstanding scientists. Howard Berg has written an account of the early history of chemotaxis (see ref. 3), and Physics Nobel Laureate Edward Purcell has written a delightful account of bacteria living at low Reynolds number, where inertia means nothing and viscosity rules (4).

Bacteria are constantly reoriented by Brownian motion, and in the short run they cannot outswim diffusion. A bacterium swimming through water experiences drag comparable to a human swimming through tar or molasses. Propulsion by reciprocal motion – fast in the power stroke and slow on the return – simply does not work. The role of diffusion is nicely summarized in Berg's *Random Walks in Biology* (5). The diversity of means by which bacteria move through liquids or across wetted surfaces has been recently reviewed (6).

With the exception of some cyanobacteria that swim by rippling their outer membrane (7), Eubacteria and Archaea overcome the reciprocal motion problem by rotating helical flagellar filaments (8). Flagella almost certainly evolved independently but convergently in those two domains of life (9). Spirochetes hide the flagellar filaments between their inner and outer membranes, so that flagellar rotation causes the entire sinusoid or helical body to serve as the propeller (10).

Flagellar rotation is driven by an inward ion current, usually of H^+ or Na^+ , through a motor embedded in the cell membrane. In many bacteria, the rotation is bidirectional, clockwise (CW), or counterclockwise (CCW), whereas in others the speed of rotation is modulated (11). Because of their small size, bacteria cannot swim straight and must frequently readjust their trajectories to generate in a 3D random walk. Depending on the species, the cells undergo periods of smooth swimming punctuated by chaotic tumbles, sudden 180° reversals, or decreases in the rate of swimming that allow Brownian motion to reorient the cell.

Most bacteria are too small to detect differences in gradient intensity across the length or width of their bodies. The solution to this problem was worked out in the Berg (12) and Koshland (13) laboratories. Bacteria constantly monitor the concentration of specific chemicals and compare them to the concentration a few seconds earlier. This comparison requires a “memory” that constantly adapts the system to ambient stimulus levels so that future changes can be detected (14, 15).

The target of the signaling pathway is the flagellar motor, which responds to the intracellular level of the phosphorylated form of the small response regulator protein, CheY. Regardless of how the random walk is generated, the level of phospho-CheY is modulated to inhibit directional reorientation, be it via tumbles, reversals, or increases in swimming speed, when the cells are moving in a favorable direction – either up an attractant gradient or down a repellent gradient. As a result, the random walk is biased. The bias is relatively low in all but the steepest gradients, and the net movement in the gradient is typically 20% of the instantaneous swimming velocity, or less. Since bacteria swim anywhere from a few to a hundred body lengths per second, cells can still migrate purposefully at up to several millimeters per minute. In the microheterogeneous environments in which bacteria typically live, this ability confers an enormous advantage in reaching localized sources of attractants or avoiding sources of repellents.

1.2. Established Methods for Assaying Bacterial Chemotaxis

The simplicity of bacterial tactic behavior makes it amenable to study by a variety of experimental methods. Some of these monitor the movement of populations of cells, other focus on individual cells. The temporal sensing mechanism makes it possible to study cells that make no net movement in a gradient, a capability that enormously simplifies the task of delivering stimuli and monitoring responses of cells that do not keep running out of the microscope field.

Some of the assays are gratifyingly simple and within the budget and equipment inventory of any laboratory. Others require considerably more sophisticated instrumentation, although, in general, these procedures are necessary only for specialized applications. In this section we provide a brief review of the standard assays in current use.

1.2.1. Swim and Swarm Plates

Polymerized agar consists of chains of extended polymers permeated by water-filled channels. At low agar concentrations (0.25–0.4% – semisolid agar) the channels are sufficiently large that the bacteria can swim through them. In the absence of chemoeffectors, cells conduct a 3D random walk through the agar matrix much as they would in liquid and spread out randomly from the point of inoculation. Since the cells are typically growing, the result is an expanding colony that forms within the agar. This “pseudotaxis” occurs in the absence of any tactic behavior. Mutant cells that only swim smoothly get trapped cul de sacs in the agar matrix (16), and their colonies do not expand significantly faster than those of nonmotile cells. Cells that only tumble or reverse also do not make much progress.

As the colony grows it decreases the concentration of metabolizable attractants where the cell density is highest. As a result,

a spatial gradient is created, and the cells migrate outward in response to that gradient. Typically, the edge of the colony shows a sharp ring where the cells congregate in the steepest part of the gradient. With some attractants, the colony expands equally throughout the agar. If the attractant can only be metabolized aerobically, the ring may only form on the surface. For bacteria that carry out aerotaxis (17), a sharp ring of cells following oxygen gradients may form only at the bottom of the agar layer, giving a dome-shaped colony that can be confused with a tactic response to another chemical. Chemotactic rings can be documented photographically by using a “bucket of light” (18).

A variation on the method is the swarm assay (19). If the agar is more solid (0.5–0.7% for *E. coli* and *Salmonella enterica*), cells can swim through the aqueous layer that forms on the agar surface. Swarming cells typically elongate and form more flagella. Although the ability to swarm depends on having an intact chemotaxis system, swarming is not chemotactic behavior per se. Some bacteria, like *Serratia*, *Proteus*, and *Bacillus* species, are very efficient swimmers, and some bacteria swarm using lateral flagella that are distinct from the polar flagellum they use for swimming in their planktonic state.

1.2.2. Capillary Assays

The capillary assay was first described by Pfeffer in the 1880s and standardized and popularized by Adler in the 1970s (20). Some of the most fundamental discoveries about chemotaxis, like the discovery of specific cell-surface chemoreceptors (2) and the identification of attractant amino acids, sugars, and other compounds (21, 22), were made using this assay.

Chambers on the order of 1 cm² and 1 mm or less thick (the pond) with one open side are loaded with a suspension of highly motile bacteria. The bacteria must be washed by gentle centrifugation or filtration and suspended in chemotaxis buffer in order to look at responses to specific compounds. Care must be taken not to damage the flagella during washing. A 1-μm capillary, sealed at one end and filled to several mm from the open end with an attractant at the desired concentration, is then inserted into the chamber. After incubation at the desired temperature – a slide warmer is handy – for 30–45 min, the capillary is removed, the sealed end broken off, and the contents blown out into dilution buffer. Dilutions are plated on nutrient agar, and the number of the cells entering the capillary is calculated from colony counts the next day. The gradient can be directly visualized by filling the capillary with a dye, and the profile of the developing gradient has been calculated as a function of time (23). Repellents can be assayed by looking at the decrease in the number of cells that enter capillaries filled with repellents compared with the number that enter capillaries filled just with buffer, or by putting repellents

into the pond and having the capillary filled with buffer (repellent-in-pond assay) (24).

By placing alternative chemoeffectors in the pond, competition between attractants can also be assessed (25, 26). Recently, a high-throughput method for running parallel assays has been developed using a 96-well microtiter plate and a matching 96-microcapillary array (27).

1.2.3. Monitoring Movement of Bacteria in Stable Gradients

Some early work in which the migration of bacterial populations was quantified utilized glycerol-stabilized spatial gradients (28). (Glycerol at the concentrations employed, 0.5–3%, is inert as a chemoeffector.) By mixing attractants or repellents with the glycerol solutions during the formation of the gradients, virtually any profile of attractant or repellent concentrations can be achieved. The position of the bacteria is determined by measuring light scattering as a function of distance along the chamber, which in the original device had a volume of 10 mL and a length of 8 cm. Typically, the bacteria are initially uniformly distributed, and they become depleted in some regions of the gradient and accumulate in others over time. Since scans of the entire gradient can be accomplished in 1 min, the kinetics of the response can be monitored. This method showed that *S. enterica* var. *typhimurium* in a gradient of L-serine extends its runs in an up-gradient direction as much as twofold without changing the length of the runs in a down-gradient direction (29).

A recent variant of this assay involves placing bacteria in a chamber between a flowing source of attractant and a flowing sink (30). This technique produces linear gradients in which the positions of individual bacteria can be registered.

1.2.4. Tracking Individual Swimming Bacteria

The observations of *E. coli* that led to the discovery of the 3D random walk and the biased random walk were performed with an automated tracking device designed and built by Howard Berg (31, 32). Berg describes the device as follows (33): “It was a three-dimensional DC servo system. Build a mechanical stage that can rapidly move a small chamber containing a suspension of swimming cells about 1 mm in any direction; design a detector that can dissect the image of a single cell; add electronics to compute errors in position and move the stage so that the image remains locked on the detector; then write down the displacement of the chamber. This tells you the displacement of the cell relative to the medium in which it is suspended. The accelerations of the chamber are so small that the bacterium does not know that it is being manipulated.” Berg’s 3D tracker is now in the laboratory of Roseanne Ford at the University of Virginia. It is still operational, although it needs to be interfaced with a new computer.

More recently, commercially available motion analysis systems have been used to track cells (34). The parameter of motility that is measured is the rcd, the rate of change of direction, in degrees, per unit time. Bacteria are tracked in two dimensions for relatively short periods, during which intervals of swimming and bouts of reorientation can be clearly distinguished. The technique can be used to measure baseline behavior and responses to added chemicals or light. A major development was the synthesis of caged compounds that can be released by flash photolysis (35). The method can measure impulse response if the beam is narrow, since the released chemicals diffuse away rapidly, and multiple stimuli can be given if the interpulse intervals are long enough. If a broader beam is used, responses to step increases in chemoefector concentration can be observed.

1.2.5. Tethered Cell Assay

The balance of forces decrees that when flagella rotate the cell body counter-rotates. If a single flagellum is held fast, a CCW-spinning flagellum turns the cell body CW, and a CW-spinning flagellum turns the cell body CCW. Thus, if a cell is tethered to the bottom of a coverslip and viewed from above, attractants lead to periods of CW-only rotation and repellents lead to briefer periods of CCW-only rotation.

Tethered cells have conventionally been attached to a surface with antibodies raised against flagellar filaments (36). The IgG molecules bind specifically to the flagella and nonspecifically to the tethering surface, usually a glass slide or coverslip. To prevent multiple flagella from attaching, the flagella are mechanically sheared to short stubs (37). Shearing can be done at high speed in the 50-mL stainless-steel cup of a kitchen blender or by passing cells back and forth rapidly between two syringes with small-gauge needles connected by thin tubing.

The fractions of total time spent in CW or CCW rotation give a quantitative measure of the run-tumble bias of a cell. This can be done using a tunnel slide, in which a chamber is formed between a microscope slide and a coverslip bridged across two other coverslips. Apiezon L grease is convenient for securing the coverslips to the slide and each other. Once the chamber is filled with cells at about 10^7 /mL and a suitable dilution of antibody, the slide is inverted and the bacteria are allowed to settle onto the coverslip for 30 min. A good preparation yields several dozen rotating cells per microscope field at $1,000\times$ magnification. Use of an *E. coli* strain that produces “sticky” filaments because of the *fliCST* mutation (38, 39) eliminates the need for antibody. A variation of this method utilizes cells that are immobilized on a surface but have a freely rotating flagellum attached to an antibody-coated latex bead (40, 41).

Many fields of cells can be video recorded and then analyzed at leisure, either by an observer with a stopwatch or by various

automated systems (42), including motion analysis devices (35). If the cells are tethered to a coverslip placed on a flow cell (43), responses to the addition or removal of attractants and repellents can also be monitored, and even modest changes of rotational bias in response to shallow temporal gradients can be measured (44). Furthermore, responses of tethered cells to the induction of proteins under control of regulatable promoters can be observed in real time (45, 46).

1.2.6. Microfluidic Assays

The unique features of microfluidics, such as small volume and large surface-to-volume ratio, laminar flow, high throughput, and compact system size for fast and accurate analysis of samples, are increasingly useful in biology. The studies that microfluidic techniques enable were previously impossible or very challenging to perform. One example is the generation of gradients of signaling molecules in microfluidic channels for investigating bacterial and eukaryotic cell chemotaxis. These devices generate concentration gradients using laminar flow phenomena in microfluidic channels to split and recombine liquids coming into the channel from multiple outlets (47–49). Such microfluidic systems can dilute or mix liquids into linear or logarithmic concentration gradients perpendicular to the fluid flow. The sampling of the gradients generated is simply determined by splitting the output from the main channel into multiple outlets. Microfluidic gradient generators have been extensively used for investigating neutrophil migration, neural stem-cell differentiation, and hepatocyte gene-expression profiling (50).

Mao et al. (51) were the first to investigate bacterial taxis in a microfluidic flow cell, in which a concentration gradient was formed by diffusion of two parallel streams. These authors showed that a significant chemotactic response to L-aspartate can be obtained with concentrations as low as 3.2 nM. In addition, it was also observed that the same molecule (L-leucine) can be sensed as an attractant by Tar and as a repellent by Tsr. Different variations of this device, such as the three-channel microfluidic device where a linear gradient is generated in the absence of flow, have been developed for investigating chemotaxis. Several microfluidic devices for investigating bacterial chemotaxis have been developed recently. One approach generates stable 2D and 3D concentration gradients using a microfluidic ladder chamber (52). Another involves using a T-shaped channel device to investigate chemotaxis perpendicular to the direction of fluid flow (53). The power of microfluidics is clearly evident from a recent demonstration of bacterial migration in response to nutrient patches with environmentally realistic dimensions and dynamics (54). These studies clearly show the potential of microfluidic approaches for investigating bacterial chemotaxis, especially in response to concentration gradients of different strengths, and for responses to two opposing gradients.

Table 1 summarizes the advantages and disadvantages of established chemotaxis assays, including microfluidic methods. In the following sections, we discuss methods for developing two new types of microfluidic chemotaxis systems: (1) a μ Plug model

Table 1
Advantages and disadvantages of chemotaxis assays

Method	
Advantages	Disadvantages
<i>Chemotactic ring formation in semisolid agar</i>	
Easy to prepare	Requires metabolizable chemoeffector
Requires minimal equipment	Hard to quantify because of metabolism
Strains can be compared directly	Adaptation masks differences in response
Mutants and revertants easily identified	Obscures differences among individual cells
	Repellent taxis difficult to observe
<i>Capillary assay</i>	
Gives quantitative data	Time consuming to prepare and score
Requires minimal equipment	Uses lots of Petri dishes and media
Gradients created by diffusion, not metabolism	Not ideal for studying repellent taxis
Many compounds can be tested in parallel	Obscures differences among individual cells
Competition between chemoeffectors can be studied	
<i>Monitoring movement of bacteria in stable gradients</i>	
Gradients are constant over time	Only one strain and condition monitored per assay
Gradients of any profile can be created	Requires significant set-up time and equipment
Kinetics of the response can be followed	Obscures differences among individual cells
Large number of cells gives good population data	
Pseudotaxis and aerotaxis not complications	
Can use gaseous chemoeffectors (e.g., oxygen)	
<i>Tracking individual swimming bacteria</i>	
The behavior of single cells can be followed	The 3D tracker is a custom-made, unique device
Can couple with photolysis of caged compounds	Motion analysis records only 2D behavior
Impulse and step responses can be measured	Motion analysis only measures run-tumble bias
	Motion analysis systems are relatively expensive
	Time consuming to collect data for statistical tests

(continued)

Table 1
(continued)

Method	
Advantages	Disadvantages
<i>Tethered cell assay</i>	
Simple to carry out	Chemotactic migration not actually measured
CW and CCW intervals determinable for many cells	Flow cells relatively hard to make and operate
Quantitative measure of performance of one flagellum	Need good microscope and video recorder
With flow cell can record responses to step gradients	Only convenient to use with large step stimuli
Can record responses to attractants and repellents	Only some types of flagellation allow tethering
Same cells can be exposed to multiple stimuli	Usually need specific anti-flagellar sera
Can look at behavior of nonchemotactic mutants	
<i>Microfluidic assays</i>	
Easy to fabricate and operate	Requires access to microfabrication facilities
High degree of reproducibility	May not work for all bacteria, as current application requires presence of GFP plasmid
Give quantitative data	
Gradients can be created over any concentration range	Cannot be used for bacteria with low motility
High throughput	

for rapid and quantitative investigation of chemotaxis under static (no-flow) conditions, and (2) a μ Flow model for investigating bacterial migration in response to concentration gradients. The former is simple enough to be carried out using either microfabrication or commonly available lab material, while the latter requires microfabrication facilities for device development.

2. Materials

2.1. Microfluidic Device

1. Commercially available hot plates.
2. Programmable spin coater (e.g., model WS-650S, Laurell Technologies Corp., North Wales, PA).

3. Mask aligner for contact lithography (e.g., model Q4000 MA, Neutronix-Quintel, Morgan Hill, CA).
4. Photolithography mask (*see* **Note 1**).
5. AutoCAD (Autodesk) or equivalent CAD program.
6. Oxygen plasma asher (e.g., model CS-1701, March Plasma Systems, Concord, CA).
7. Polished silicon wafer.
8. SU-8 photoresist (Microchem, Newton, MA).
9. SU-8 developer (Microchem, Newton, MA).
10. Acetone.
11. Isopropanol.
12. Polydimethylsiloxane (PDMS): Slygard 184 (Ellsworth Adhesives, Germantown, WI).
13. Tygon tubing.
14. Microscope slides.
15. Blunt 20-gauge needles.
16. Blunt 30-gauge needles.
17. Picoplus pump (Harvard Apparatus, Boston, MA).
18. 500- μ L glass syringes (Hamilton, Reno, NV).
19. 50- μ L glass syringe (Hamilton, Reno, NV).
20. Bromophenol blue.
21. Low-melting temperature agarose.
22. 1.5-mm biopsy punches.

2.2. Microscopy and Imaging

1. Leica TCS SP5 Resonant Scanner Confocal or equivalent fast imaging fluorescent microscope.
2. Matlab V7 (Mathworks, Natick, MA).

2.3. Bacteria, Plasmids, Culture Media, and Buffers

1. *E. coli* RP437 (**55**).
2. Green Fluorescent Protein (GFP) expression plasmid: pCM18 (**56**).
3. Red Fluorescent Protein (RFP) expression plasmid: pDsRed-Express (Clontech, Palo Alto, CA).
4. Phosphate-buffered saline (PBS): 8.71 g/L NaCl, 2.17 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.26 g/L KH_2PO_4 ; Dissolve in 800 mL H_2O , adjust pH to 7.4, and bring volume up to 1 L.
5. Sterile tryptone broth (TB): 10 g/L tryptone, 8 g/L NaCl.
6. Sterile chemotaxis buffer (CB): 1 $\times$ PBS, 0.1 mM EDTA, 0.01 mM l-methionine, and 10 mM DL-lactate.
7. 0.4- μ m HTTP Isopore membrane filters (Millipore, Billerica, MA).
8. Sterile 50-mL centrifuge tube (Corning, Corning, NY).

3. Methods

3.1. Microfluidic Device Design

Schematics of the static (μ Plug) and flow-based (μ Flow) chemotaxis devices are shown in **Fig. 1**. The μ Plug chemotaxis device is a microfluidic version of the well-established plug-in-pond method (24, 57). In this device, a plug of agarose containing the chemoeffector molecule is formed in a square, microfabricated chamber (15 mm sides and $\sim 75\ \mu\text{m}$ high). Agarose is introduced through a 1.5-mm diameter hole in the middle of the chamber, and two 1.5-mm holes along the diagonal serve to introduce cells into the chamber and to provide a vent, respectively.

The μ Flow device is a flow-based system that consists of two modules – one for generation of concentration gradients and the second for observing chemotaxis. The first section consists of a diffusive mixing-flow system that generates the concentration gradients. Two fluid flow streams containing chemoeffectors at different concentrations are brought together and mixed before being split into three streams. Since flow is laminar, mixing takes place only through diffusion. This mixing and splitting process

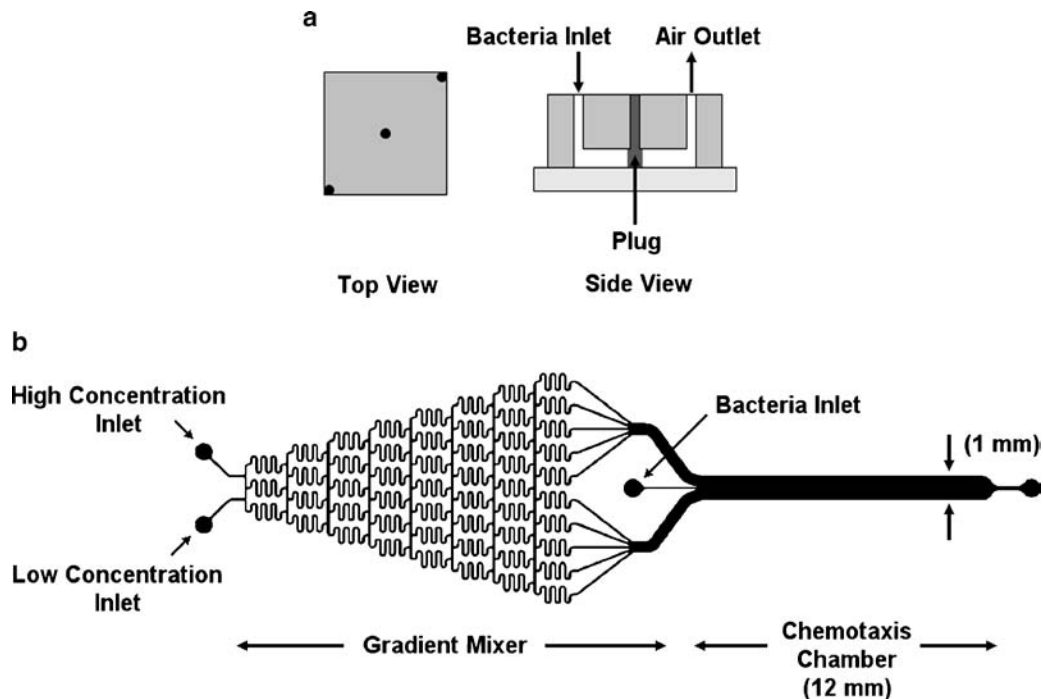


Fig. 1. Microfluidic chemotaxis device designs. (a) *Top and side views* of the μ Plug model are shown. (b) Schematic of the μ Flow gradient chemotaxis device in which a linear concentration gradient of signaling molecules can be generated in the microfluidic channels through diffusion. Bacteria are introduced through the *middle inlet*.

is repeated multiple times (e.g., three streams from the first segment are mixed to generate four streams, etc.) until outlet streams with the desired number of concentrations are formed. While the system described here is for generating a linear gradient between two inlet concentrations, different gradient profiles (e.g., exponential gradients) can be generated at the outlet by using multiple inlets and/or altering the flow characteristics (58). The gradient generator connects to a downstream flow chamber for investigating bacterial chemotaxis. Bacteria enter the flow chamber in the middle of the gradient and move toward or away from higher concentrations of chemoeffector, depending on whether it is an attractant or a repellent. Inlet and outlet ports for bacteria are punched using a blunt 20-gauge needle whose edges are beveled using very fine grit sandpaper.

3.1.1. Generation of Device Molds

A typical protocol for producing molds for microfabricated devices is given as follows.

1. Draw device design in AutoCAD or equivalent CAD software.
2. Use the CAD file to create the photolithography mask in a high-resolution printer. Obtain photolithography mask (*see Note 1*).
3. Prepare silicone wafer by rinsing it with acetone, followed by isopropanol. Dry the wafer gently using a dry air or nitrogen stream. Place dried wafer on a hot plate set to 65°C.
4. Select the appropriate SU-8 required for achieving the desired feature height of the device. Coat the wafer with SU-8 in a spin coater using the appropriate spin speeds and times for the corresponding feature height (*see Note 2*).
5. Place the spin-coated wafer on the hot plate at 65°C followed by 95°C. The pre-exposure (“soft”) bake times are determined by the thickness of the coated SU-8 (*see Note 2*). For example, a bake time of 3 min at 65°C and 6 min at 95°C is used for the μ Plug device which has a height of $\sim 75\ \mu\text{m}$.
6. Expose the spin-coated wafer to UV light using the photolithography mask and the mask aligner. The exposure time is determined by the thickness of the SU-8 (*see Note 2*).
7. Place the spin-coated wafer on the hot plate at 65°C followed by 95°C. The postexposure (“hard”) bake times are determined by the thickness of the film (*see Note 2*).
8. Develop the wafer by submerging it in SU-8 Developer. The development time is determined by the thickness of the SU-8. Rinse wafer with isopropanol (*see Note 2*).
9. Place developed wafer on a hot plate at 120°C for 30–60 min. This finished wafer is known as the “master” mold.

3.1.2. Device Fabrication

1. Pour PDMS to desired thickness over mold. Degas PDMS to remove any air bubbles.
2. Cure PDMS by heating on a hot plate. Various combinations of temperature and time can be used. In general, higher temperatures require less heating time. Our lab uses 80°C for 2 h. For thicker PDMS layers, more time may be required.
3. Remove PDMS from mold by peeling PDMS away from the mold. The desired structure will be embedded in the PDMS block.
4. Punch holes in PDMS (*see Note 3*).
5. Clean glass microscope slide with isopropanol and dry with a dry nitrogen or air stream.
6. To bond the PDMS and glass slide, first treat both with oxygen plasma in the plasma asher. Next, bring the PDMS into contact with the glass slide. Lastly, heat the contacted slide and PDMS on a hot plate set to 65°C for 15 min.

The scheme for making the master mold and device fabrication scheme is shown in **Fig. 2**.

3.2. Growth of Highly Motile *E. coli*

1. Grow overnight cultures of the bacterial strains of interest in 5 mL of TB medium with the appropriate antibiotic at 32°C, with shaking (*see Note 4*).
2. Use the overnight culture to inoculate a new culture in a 250-mL Erlenmeyer flask containing 20 mL of TB medium with the appropriate antibiotic (*see Note 5*). The starting optical

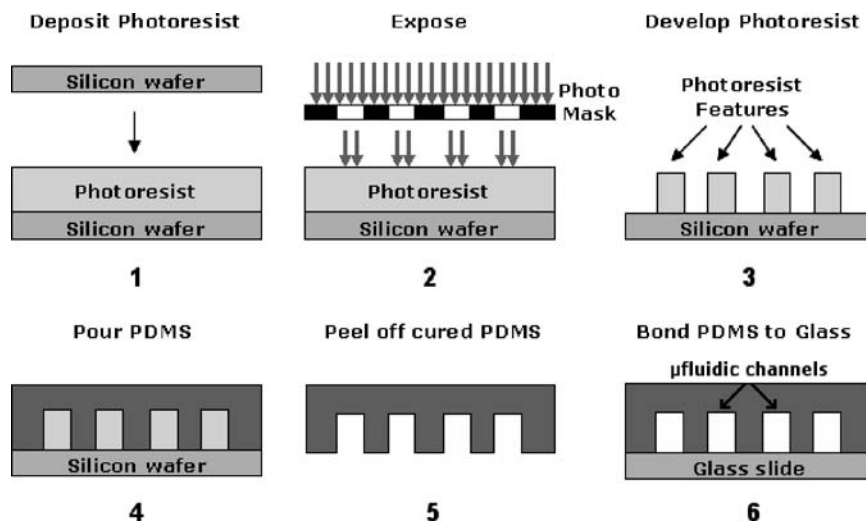


Fig. 2. Outline of steps involved in the generation of the silicon-wafer master mold and fabrication of microfluidic devices. Photolithography techniques are used to generate a silicon-wafer master mold patterned with the negative image of the desired microfluidic pattern. A PDMS mold is made from the silicon-wafer mask and irreversibly bonded to a glass slide to generate a microfluidic flow cell.

density at 600 nm, OD_{600} , should be approximately 0.05. Grow the culture with shaking at 32°C.

3. At an OD_{600} of ~0.7, harvest the cells by filtering 1–3 mL of culture through a 0.45- μ m pore-diameter nitrocellulose filter (*see Note 6*). Wash the cells with 15 mL of chemotaxis buffer (CB). Place the filter paper gently into the bottom of a 50-mL Falcon tube and resuspend the cells in 1–2 mL of CB by very gentle shaking (*see Note 7*).
4. Keep the cells at 32°C and use within 30 min, or for longer storage keep cells on ice and then warm before use.

3.3. μ Plug Assay

1. For the plug, dissolve 20 mg of low-melting-temperature agarose in 950 μ L of CB. Add 50 μ L of saturated bromophenol blue solution. Melt the agarose at 70°C.
2. Reduce the temperature to 55°C and add the chemoeffector to the desired final concentration.
3. Mix thoroughly and add 5 μ L of the agarose mixture to a prewarmed μ Plug device via the center hole.
4. Let the device cool to room temperature for 2–5 min.
5. Mix GFP-expressing and RFP-expressing cells so that you have the same density of green-fluorescing and red-fluorescing cells in the suspension and introduce the cell suspension into the chamber via one of the corner holes until full. Avoid air bubbles.
6. Place the device on the stage of a fluorescent microscope and image cells at different time intervals. We typically image cells immediately after the device is placed on the microscope stage and subsequently every 5 min for 20 min (*see Note 8*). If time-lapse movies are to be made, image cells more frequently (every 1–2 min).

3.3.1. Results with the μ Plug Device

The formation of a fluorescein concentration gradient in the μ Plug device is shown in **Fig. 3**. Fluorescein was added to the agarose plug at a concentration of 100 ng/mL, and uninoculated CB was added to the device. The device was imaged using a 5 $\times$ objective every minute for 20 min. The pixel intensity was used to determine the concentration profile over time.

The migration of *E. coli* RP437 in the μ Plug device in response to $NiSO_4$ and L-serine is shown in **Fig. 4**. For a repellent, a band of GFP-labeled bacteria moves away from the plug as Ni^{2+} diffuses into the CB. For strong repellents, like Ni^{2+} , this repellent band forms and moves quickly (i.e., within ~15 min). For an attractant, the bacterial band becomes brighter and thicker as time progresses. Depending on the concentration and efficacy of the attractant being tested, this response can also occur rapidly.

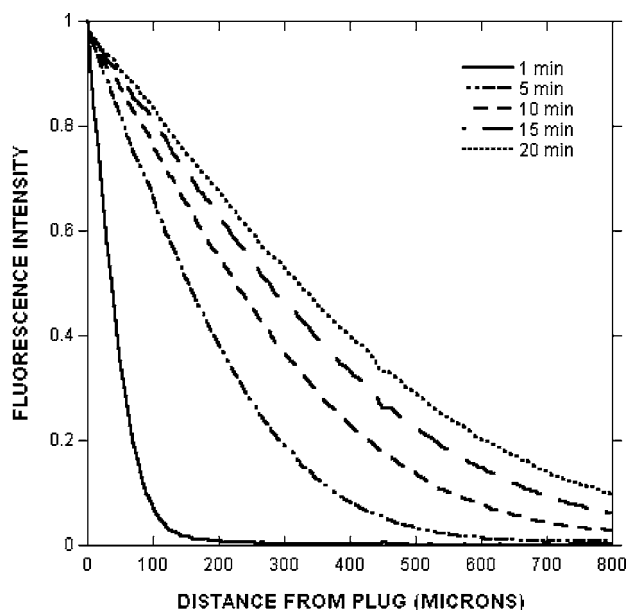


Fig. 3. Characterization of gradient formation in the μ Plug device. The diffusion of 100 ng/mL fluorescein from the μ Plug was monitored using fluorescence microscopy for 30 min. Fluorescence images were quantified by image analysis and used to determine the distance to which the fluorescent dye diffuses (i.e., the concentration gradient) surrounding the plug.

3.3.2. Data Analysis: μ Plug

The advantages of the μ Plug method are the rapid identification of the chemotactic response to a molecule and the ability to compare the responses of two different bacteria using differential labeling (i.e., one strain expresses GFP while the other expresses RFP). In Fig. 4a and b, the RFP-labeled cells were killed by exposure to a lethal dose of kanamycin to provide a nonresponsive control to monitor bulk flow, which was negligible. However, this method does not lend itself to quantitative assessment of migration. To obtain a more quantitative characterization of chemotaxis, the flow-based microfluidic device described in the following section was developed.

3.4. Microfluidic Chemotaxis (μ Flow)

Our microfluidic flow approach is based on the technique of Mao et al. (51). In that system, bacteria are introduced between two parallel streams of buffer, one containing the chemoeffector, the other without added chemical. The gradient forms by diffusion of the chemoeffector as the parallel laminar flow streams move down the channel. If a response to steep, step-wise gradients of chemoeffectors is desired, our molds can easily be modified to generate that flow profile.

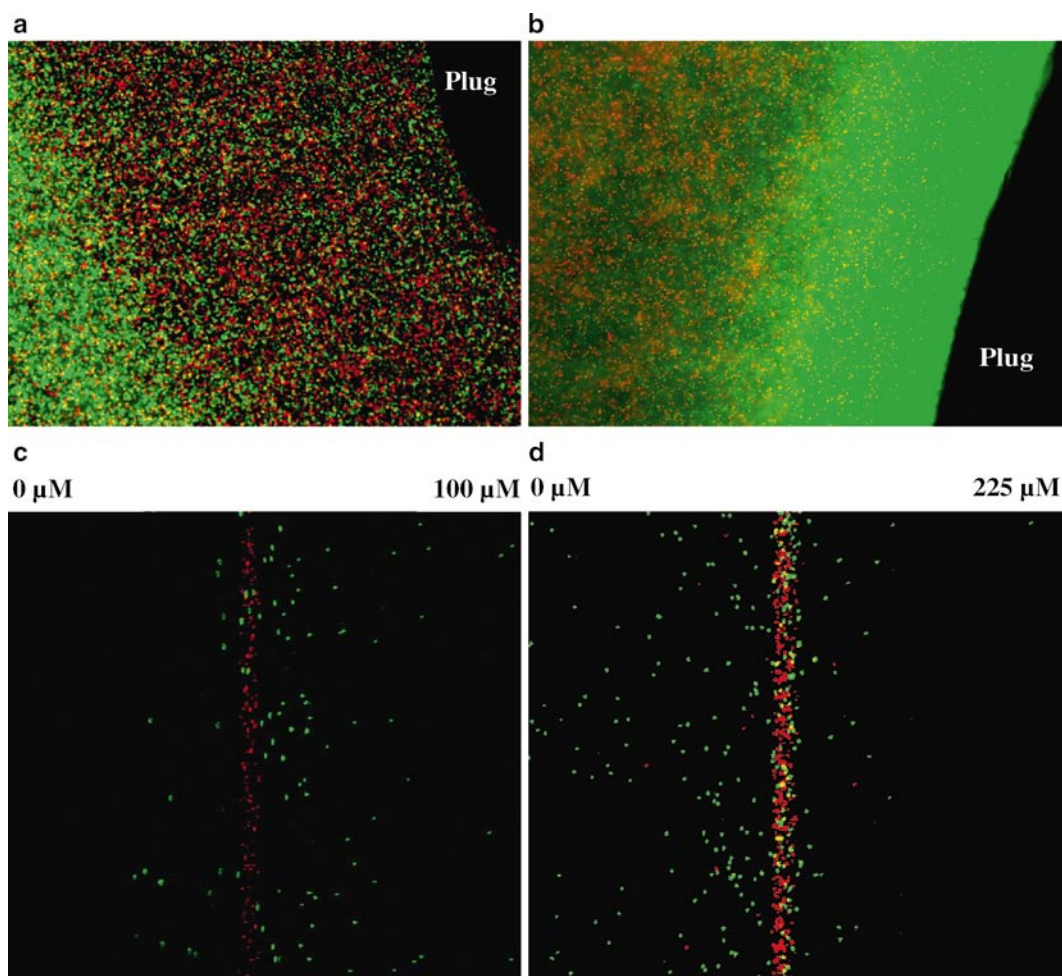


Fig. 4. Fluorescent images of chemotaxis by *E. coli* strain RP437 in the μ Plug and μ Flow devices. In the μ Plug device, bacteria were exposed to: (a) 225 μ M NiSO₄; (b) 100 μ M L-serine. Cells were imaged after 30-min incubation at room temperature. The location of the agarose plug containing the chemoeffector molecule is indicated. Live bacteria are *green*, dead bacteria are *red*. The repellent and attractant bands, respectively, of bacteria in response to NiSO₄ and L-serine are clearly visible. Data shown are representative images from three independent experiments. In the μ Flow device, *E. coli* RP437 was exposed to a gradient of (c) 0–100 μ M L-aspartate; (d) 0–225 μ M NiSO₄. The migration of live bacteria toward L-aspartate or away from Ni²⁺ was imaged every 2.5 s and quantified by image analysis. Data shown are representative images from three independent experiments.

Prepare the μ Flow device (**Fig. 5**) using the methods described for the μ Plug device in **Subheading 3.1.2**.

1. Cut tubing to the correct length for your device setup (*see Note 9*).
2. Using forceps, insert one end of tubing into the device.
3. Using forceps, insert the blunt 30-gauge needle into the other end of the tubing.
4. Using a 3-mL syringe, collect 1 mL of CB. Remove any air bubbles from the syringe.

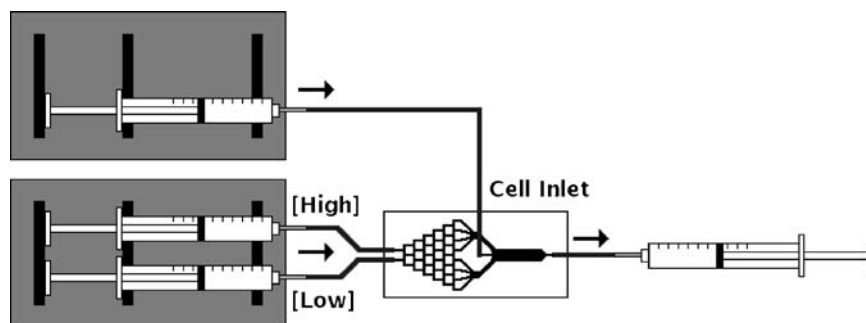


Fig. 5. Setup for the μ Flow device. The chemoeffector to be tested is introduced at two concentrations (high and low) into the gradient-generation (upstream) portion of the device. Bacteria are introduced into the device via the central inlet. The entire device is mounted on a resonant-scanning confocal microscope for continuous imaging.

5. Add CB to the needle hub on the outlet tubing. Tap the needle hub to remove any air bubbles.
6. Push a little CB out of the tip of the syringe and connect the 3-mL syringe to the needle hub without trapping air.
7. Push CB through the microfluidic gradient device until you have filled all remaining needle hubs (*see Note 10*).
8. Fill the 500- μ L syringes with CB containing the chemoeffector being tested. Make sure there are no air bubbles in the syringe.
9. Push out a little of the syringe contents so that you have just a little drop on the end of the syringe. Touch this drop on the tip to the liquid in the needle hub and attach. This method will reduce the chances of trapping air bubbles in the needle hub (*see Note 11*).
10. Prepare GFP-labeled cells as mentioned previously with the following modifications: In **step 3** of cell preparation, harvest the cells at an OD_{600} between 0.35 and 0.55. Resuspend cells to an OD_{600} of 0.35 using CB containing the chemoeffector at the concentration expected in the middle of the channel (*see Note 12*). Also add RFP-labeled dead cells at an OD_{600} reading of 0.35 to test the laminar flow characteristics.
11. Remove some of the CB from the cell inlet needle hub. Refill with the resuspended cell mixture.
12. Gently fill the 50- μ L syringe with the resuspended cells (*see Note 13*).
13. Using the method described previously, attach the 50- μ L syringe to the inlet-needle hub.
14. Set the device on the microscope stage. Place the inlet syringes into the syringe pump. Select your flow rates and run the syringe pumps (*see Note 14*).
15. Once you see cells enter the flow chamber, wait ~ 20 min for the system to stabilize before imaging. We collect 100

images at an interval that allows for cells at the top of the first image to be captured at the bottom of the second image. This time will vary based on the flow rate (*see* **Note 14**).

3.4.1. Results: μ Flow

The concentration gradient formed in the μ Flow device is shown in **Fig. 6**. The cell inlet was capped so that there was no flow through it. The high-concentration syringe contained CB with 100 ng/mL of fluorescein. The low-concentration syringe contained CB only. The syringe pumps were started and time was allowed for a stable gradient to form. The chemotaxis chamber was then imaged near the outlet using a $5\times$ objective. The pixel intensity was used to determine the concentration profile. **Fig. 4c** and **d** shows fluorescence images of migration in attractant and repellent, respectively, gradients in the μ Flow device.

3.4.2. Data Analysis: μ Flow

The images obtained from the μ Flow device yield a quantitative measure of chemotaxis. The main steps involved in image analysis, using Matlab version 7, are outlined later. Details about the Matlab code and the protocol for image analysis are available upon request.

Based on the quantified images, the chemotaxis partition coefficient (CPC) and the chemotaxis migration coefficient (CMC) can be calculated as described in (51). If a cell is detected on the high-concentration side (it migrates to the right), it is given a value of +1, whereas a cell detected on the low-concentration

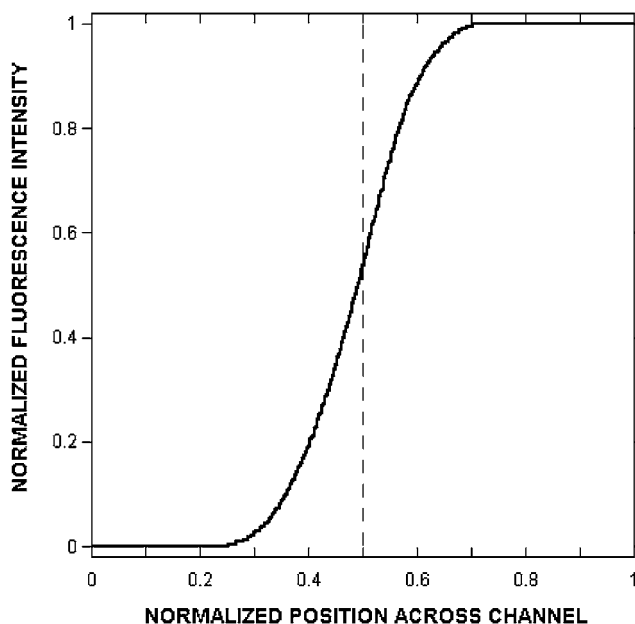


Fig. 6. Characterization of gradient formation in the μ Flow device. A concentration gradient between 0 and 100 ng/mL fluorescein was generated in the μ Flow device and imaged using fluorescence microscopy. Fluorescence images were acquired after 30 min and quantified by image analysis.

side is given a value of -1 . The values are summed up and divided by the total number of cells to generate the CPC. Depending upon the sign of the CPC (positive or negative), the direction of migration can be determined (toward or away from a signal).

Although the CPC indicates whether cells respond to a chemical as an attractant or a repellent, it does not quantify the chemotactic response. The CMC measures the strength of the response. The CMC is calculated by dividing the spatial distribution profile of bacteria (across the width of the device) into 64 sections (32 on each side of the cell inlet). Using the same notation as in the CPC calculation, cells on either side of the inlet are assigned values of $+1$ or -1 . This value is then multiplied by a weighting factor based on the distance of migration. The sections farthest from the center (sections 1 and 64) are multiplied by 1 ($=31.5/31.5$) since they contain cells that have migrated the maximum distance. The next two sections (2 and 63) are multiplied by 0.968 ($=30.5/31.5$). The last two sections (i.e., 32 and 33 that are closest to the cell inlet) are multiplied by 0.015 ($=0.5/31.5$). A CMC of $+1$ indicates that all the bacteria moved completely up the gradient to the wall of the flow chamber. In the case of a mild attractive response, the CMC would still be positive, but have a smaller value than the CPC. Thus, the CMC discriminates responsive cells based on the extent of migration. Fig. 7 shows CPC and CMC values for *E. coli* RP437 chemotaxis

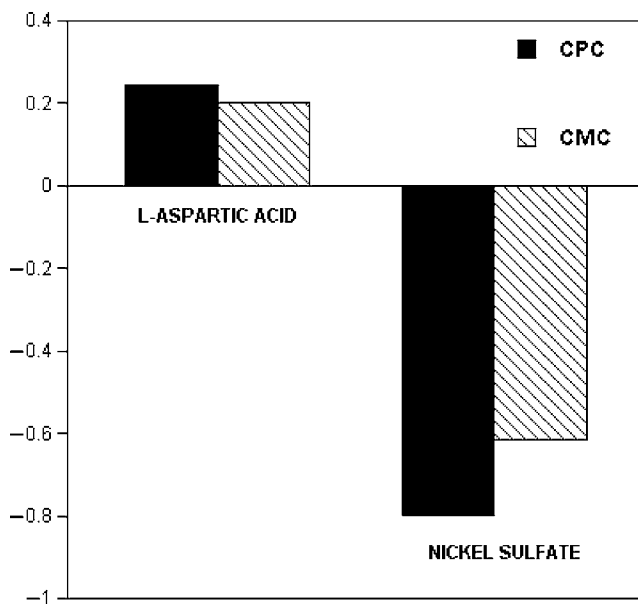


Fig. 7. Quantification of *E. coli* RP437 chemotaxis in μ Flow devices. Bacterial responses to gradients of 0–100 μ M L-aspartate and 0–225 μ M NiSO_4 were quantified using image analysis. The data were used to calculate the chemotaxis partition coefficient (CPC) and chemotaxis migration coefficient (CMC). The CPC and CMC values shown correspond to the images shown in Fig. 4c and d.

in response to L-aspartate and NiSO_4 for the gradients shown in Fig. 4c and d.

We anticipate that the μFlow device can be used for enrichment and isolation of mutants that are incapable of recognizing specific attractants and repellents, by the simple expedient of collecting cells from the side of the channel that is not frequented by the wild-type bacteria. This approach should not select for nonmotile or nonchemotactic mutants, since they will remain near the middle of the channel. This technique should also be applicable for identifying bacteria in environmental samples that respond to particular chemicals as attractants or repellents.

4. Notes

1. Photolithography masks can be printed at Stanford Microfluidics Foundry (<http://thebigone.stanford.edu/foundry/>). Device molds, as well as complete devices, can be created from the Stanford Microfluidics Foundry.
2. More information on SU-8 and the various processing times based on thickness are available at the manufacturer's product information web page (http://www.microchem.com/products/su_eight.htm).
3. For the μPlug device, use 1.5-mm biopsy punch to make the holes in the PDMS. For the μFlow device, punch the holes using the blunt 20-gauge needle.
4. Grow *E. coli* cells at 32°C for best motility. Higher temperatures reduce motility. The media used for growth is important. Using the wrong media can poison or reduce the motility of the bacteria. All our experiments used tryptone broth.
5. For certain receptors to be present in bacteria, it may be necessary to grow the bacteria in the presence of an inducing chemoeffecter; e.g., galactose, ribose, and maltose for *E. coli*. For these sugars, add at 0.1% (w/v).
6. For generating the dead RFP-expressing cells, grow *E. coli* containing plasmid pDSRed to an OD_{600} of ~0.7 and add kanamycin to a final concentration of 1 mg/mL. After 1 h, wash the cells in CB. These cells may be stored at 4°C.
7. Gentle shaking/rolling of the tube is required. Excessive shaking can shear the flagella and reduce the motility of the sample.

8. For less-motile bacteria, longer imaging times may be needed. The advantage of the PDMS device is that it greatly reduces evaporation and allows for extended imaging on the microscope.
9. For easier insertion of needles into tubing, cut tubing at a 45° angle, using a razor blade.
10. It is very important to flush the device with CB from the outlet port. Otherwise, air bubbles will be trapped in the gradient mixer. If you do get air bubbles, you can remove them by creating pressure in the device. This pressure will push the air through the gas-permeable PDMS. Filling from the outlet should minimize the number of bubbles.
11. Air bubbles must be removed, as any air bubble trapped in the needle hub, or in the syringe, can come loose under flow and become trapped in the device. This will disrupt the profile gradient so that the experiment will need to be repeated.
12. To obtain the correct concentration of chemoeffector in your cell resuspension, knowledge of the concentration profile exiting the gradient mixer is required. For a linear gradient, the correct concentration is halfway between the low and high concentrations. For example, if your low is 0 μM and your high is 100 μM , the chemoeffector concentration in the cell resuspension should be 50 μM .
13. To avoid shearing off the flagella, be very gentle. Otherwise, swimming motility can be greatly reduced.
14. Optimal flow rates may vary based on the swimming speed of your bacteria. Slower flow rates may be required for low motility bacteria. The optimal flow rate for your sample must be determined empirically. In all cases, you should use a population with the best motility achievable for your strain.

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Chapter 2

Prokaryotic Phototaxis

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Summary

Microorganisms have various mechanisms at their disposal to react to (changes in) their ambient light climate (i.e., intensity, color, direction, and degree of polarization). Of these, one of the best studied mechanisms is the process of phototaxis. This process can be described as a behavioral migration-response of an organism toward a change in illumination regime. In this chapter we discuss three of these migration responses, based on swimming, swarming, and twitching motility, respectively. Swimming motility has been studied using a wide range of techniques, usually microscopy based. We present a detailed description of the assays used to study phototaxis in liquid cultures of the phototrophic organisms *Halobacterium salinarum*, *Halorhodospira halophila*, and *Rhodobacter sphaeroides* and briefly describe the molecular basis of these responses. Swarming and twitching motility are processes taking place at the interface between a solid phase and a liquid or gas phase. Although assays to study these processes are relatively straightforward, they are accompanied by technical complications, which we describe. Furthermore, we discuss the molecular processes underlying these forms of motility in *Rhodocista centenaria* and *Synechocystis* PCC6803. Recently, it has become clear that also chemotrophic organisms contain photoreceptor proteins that allow them to respond to their ambient light climate. Surprisingly, light-modulated motility responses can also be observed in the chemotrophic organisms *Escherichia coli* and *Acinetobacter calcoaceticus*. In the light-modulated surface migration not only “che-like” signal transduction reactions may play a role, but in addition processes as modulation of gene expression and even intermediary metabolism.

Key words: *Halorhodospira halophila*, *Ectothiorhodospira*, *Halobacterium salinarum*, *Rhodobacter sphaeroides*, *Synechocystis*, *Rhodocista centenaria*, *Rhodospirillum centenum*, Swimming motility, Swarming motility, Twitching motility, Photoactive yellow protein, Sensory rhodopsin, Phytochrome, BLUF, Redox sensing

1. Introduction

Prokaryotic microorganisms have various modes at their disposal for their own dispersal, as well as to be able to be attracted and/or repelled by a wide range of abiotic and biotic signals. The best known of these is the swimming behavior, relevant in aqueous environments, in which cells are propelled by one or more rotating flagella. Other types of migration in this environment are based on floatation, i.e., on the difference in density between the cells and their surroundings, which can be affected by, e.g., glycogen synthesis and the formation or collapse of gas vesicles.

Besides in the aqueous phase, bacteria can migrate also on the interface between a solid phase on the one hand and a liquid phase or a gas phase on the other, in a multitude of ways. These have been referred to in literature with terms as: swarming, gliding, twitching, sliding, etc. (1). Only for swarming and twitching motility has the molecular basis for the particular mode of migration been satisfactorily resolved. Swarming cells are propelled also by rotating flagella, but these latter organelles during swarming are often much more numerous, expressed on a per-cell basis, and in addition they are peritrichously inserted into the cytoplasmic membrane. In twitching motility the displacement of the cells is effected by expanding and contracting surface appendages, called pili (2), usually referred to as Type IV pili).

Besides random migration for dispersal, many motile bacteria can also migrate in a specific direction, steered by environmental signals by which they are attracted or repelled. The prototype system in this respect is the chemotaxis (Che) system from enterobacteria like *Escherichia coli*, which allows organisms to show both excitability and adaptation. This system uses: (1) multiple extracellular and intracellular (ligand) sensing domains (mostly organized in a large array in one of the poles of the cell) as part of the so-called methyl-accepting chemotaxis proteins (MCPs), (2) two-component system-based phosphoryl transfer reactions to activate at least one small response regulator that modulates flagellar rotation and in addition a methyl-esterase, and (3) methanol release as a reporter of the adaptation process (see ref. 3 for a review). All three types of motility discussed here (i.e., swimming (3), swarming (4), and twitching (5) motility) can become directional by signal input from a Che-like signal transduction system (see also Fig. 1 and (6)).

The ability to migrate in space is important not only for the microorganism, but also for plant and animal hosts that may suffer a pathogen's infection. Ample evidence has been provided that the ability to migrate correlates with the virulence of many pathogenic bacteria (see e.g. (2, 7, 8) for some recent references).

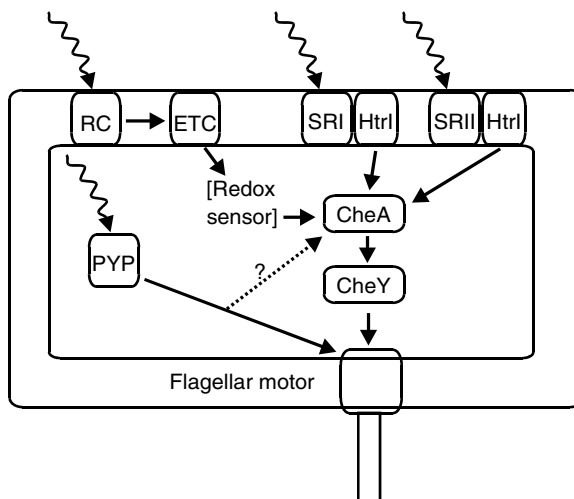


Fig. 1. Summary of available knowledge on the phototaxis signaling pathways in *H. salinarum*, *R. sphaeroides*, and *H. halophila* in a Che-like reaction scheme. *H. salinarum* contains the photoreceptors SRI and SRII, which are complexed in the membrane to their signal transducers HtrI and HtrII. These transducers modulate the autokinase activity of CheA and thus modulate the phosphorylation status of CheY. Phototaxis of *R. sphaeroides* proceeds via its photosynthetic reaction center (RC) and electron transfer chain (ETC) via a putative redox sensor. Positive phototaxis in *H. halophila* occurs via a similar pathway, while its negative phototaxis is triggered by photoactive yellow protein (PYP). The signal transduction pathway for PYP is unknown; one candidate is the Che system. Possible adaptation mechanisms have been omitted from this figure.

In this contribution we will discuss the behavioral responses of bacteria in response to changes in their illumination regime, in a range of processes loosely referred to as “phototaxis.” Whereas originally this term was used to refer to a behavioral reaction of an individual organism *toward* a light source (9), we will here use the term for any cellular or multicellular migration response toward a change in the illumination regime. In this discussion, however, we will restrict our discussion to examples based on the three well-resolved modes of migration: swimming, swarming, or twitching motility. Use of the term “phototaxis” in this way implies that it is a process also affected by intermediary metabolism and modulation of gene expression.

1.1. Phototaxis in Liquid Cultures

Phototaxis has been examined in some detail in a number of photosynthetic prokaryotes (9), particularly halophilic Archaea, purple photosynthetic Proteobacteria, and Cyanobacteria (for purple bacteria and Archaea, see Fig. 1). Two very different mechanisms of photosensing are at the basis of these phototaxis responses: some responses are triggered via light absorption by the photosynthetic machinery, while others involve dedicated photosensory proteins.

Interactions and spectral overlap between these two modes of sensing provide another layer of complexity in these photosensory pathways. The final output in terms of motility is also varied: for free-swimming bacteria it ranges from light-induced changes in swimming direction to transient stops by attractant removal, and in bacteria moving on a solid surface from gliding of entire bacterial colonies to changes in twitching motility. Only for phototaxis in halophilic Archaea is detailed information available on the entire photosensory signaling pathway.

The motility of *Halobacterium salinarum* (previously *Halobacterium halobium*) is based on its five to eight flagella, placed in tufts at the cell poles (10–12). It swims equally well in both directions (clockwise and counterclockwise rotation of the flagellum) along its long cell axis, at a relatively slow speed of approximately 2.5 $\mu\text{m/s}$, and reverses its swimming direction about every 10–15 s, or sometimes stops. The duration of a typical straight run of a cell roughly corresponds to the time scale on its swimming direction will be randomized. The ability of *H. salinarum* to swim equally well in both directions, and to reverse swimming direction is different from the situation found in *E. coli*, where counterclockwise rotation of the peritrichous flagella causes the assembly of a flagellar bundle, resulting in swimming. Counterclockwise rotation results in the disassembly of the flagellar bundle, causing “tumbling” of *E. coli*. The frequency of these reversals is regulated by chemical and light stimuli. In between reversals *H. salinarum* usually swims following nearly straight paths. Highly motile strains of *H. salinarum* have been obtained by selection (13).

The functioning of the photoreceptors and signal transduction pathways that trigger phototaxis in *H. salinarum* has been analyzed to a high degree of sophistication (14, 15). Phototaxis is initiated by the archaeal rhodopsins sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII; see Fig. 1). These retinal-containing proteins membrane proteins are complexed to signal transducer proteins, strongly resembling the methyl-accepting chemotaxis proteins (MCPs) that serve as the receptors for chemotaxis in *E. coli*. These halobacterial transducer proteins (HtrI and HtrII) interact with cytoplasmic signal transduction components that again strongly resemble those of *E. coli*, and that relay signals to the flagellar motor complex by changes in the phosphorylation state of CheY. The molecular mechanism of the photoactivation of SRI and SRII, and the relay of the signal from SRII to its transducer HtrII have been studied at near-atomic resolution (16). SRI functions as the receptor for positive phototaxis to orange light. In addition, its blue-shifted photocycle intermediate is the signaling state for a negative phototaxis in response to near-UV light. Sensory rhodopsin II triggers negative phototaxis toward blue light.

Rhodobacter sphaeroides (previously *Rhodospseudomonas sphaeroides*) swims by means of a single flagellum located in the center (not at the pole) of the cell (17). This single flagellum only rotates in the clockwise direction and always pushes the cell. Cells switch (typically every ~10 s) between periods of swimming (clockwise rotation of the flagellum) and periods during which the flagellum does not rotate, and the cells consequently stop swimming for approximately 1 s. Thus, the intermittent stops of *Rb. sphaeroides* are equivalent to tumbling in *E. coli*. *Rb. sphaeroides* can swim at speeds up to 80 $\mu\text{m/s}$. The duration of the periods of swimming and stalled flagellar motion is altered under the influence of external stimuli such as light. Its flagellar resetting bias in non-stimulated cells is 0.85, as opposed to the 0.5 bias in *E. coli*. By consequence *Rb. sphaeroides* responds significantly more strongly to negative stimuli than to positive stimuli (18).

Phototaxis in *Rb. sphaeroides* is triggered by the effects of the photosynthetic machinery on the rate of electron transfer (9). Thus, in contrast to the situation in *H. salinarum*, phototaxis in *Rb. sphaeroides* does not involve a dedicated photosensor. The rate of electron transfer is presumably sensed by an as yet unidentified receptor, and relayed into the complex set of Che proteins in *Rb. sphaeroides* (Fig. 1). Thus, phototaxis responses in *Rb. sphaeroides* can be regarded as a form of redox taxis and are modulated by factors affecting electron transport, such as the presence or absence of oxygen (19). The *Rb. sphaeroides* genome encodes nine transmembrane chemoreceptors (MCPs) and four putative cytoplasmic MCPs, four CheA proteins, and six CheY proteins (20). A number of proteins from this Che system have been shown to be required for phototaxis in *Rb. sphaeroides*, showing that the signal transduction chains for phototaxis and chemotaxis converge at this level (21). A similar situation holds for phototaxis and chemotaxis in *R. centenum* (see later).

Halorhodospira halophila (previously *Ectothiorhodospira halophila*) swims by means of two polar flagella. Like *H. salinarum*, it swims equally well in both directions (22). Relatively straight (or slightly circular) runs are separated by a reversal in swimming direction; occasionally cells can undergo brief stops. The rate of swimming is variable and can reach up to 100 $\mu\text{m/s}$. Attractant photostimuli increase the frequency of reversals in swimming direction, while repellent photostimuli suppress reversals for periods longer than 5 s.

H. halophila exhibits both positive and negative phototaxis (22). The positive phototaxis response is triggered through the photosynthetic machinery, as is the case for *Rb. sphaeroides*. The wavelength dependence of the negative phototaxis response has indicated that photoactive yellow protein (PYP) functions as the dedicated photoreceptor for this response. While the mechanism for the light activation of purified PYP has been unraveled in

great depth (23), the signal transduction chain linking PYP to the flagellar motor has not yet been resolved (Fig. 1).

1.2. Phototaxis on (Semi)Solid Surfaces

1.2.1. Overview

It has become clear that many organisms are capable of (photo)tactic behavior not only in liquid cultures, but also on (semi)-solid surfaces. Later, we will review current knowledge on well-studied cases of phototactic motility of bacteria on solid surfaces, mostly the surface of an agar plate but a glass surface can also be used. The general method to study this is to spot a small volume (typically 1–5 μ l) of a liquid bacterial culture in a well-defined growth phase on a low-concentration agar plate. The exact agar concentration needed for highest motility varies depending on the organism studied, but is generally 0.5–0.8%. Usually, the lowest agar concentration yields the largest colony diameter (24). Besides the concentration of agar, various additional factors can influence the rate of movement of a bacterial colony over a semisolid surface. For example, the presence of surfactants can increase motility significantly (25). Niu et al. showed that addition of 0.02% Tween80 could lead to an up to fivefold increase of colony diameter of swarming *E. coli* cells. Surfactants, either excreted by the cells themselves, or added to the soft agar, also enhance (swarming) motility (26). Also, the brand of agar used has been shown to influence motility. For example, Toguchi et al. compared motility on Eiken agar and Difco agar (26). Colony diameter was significantly larger on Eiken agar, and surfactant defects in bacterial strains could be overcome by using Eiken agar instead of Difco, due to some unknown parameter of the Eiken agar, such as superior wettability.

Furthermore, it cannot be stressed enough that these responses on solid surfaces are influenced by many external factors, apart from those mentioned earlier. Extreme care should be taken to precisely monitor and regulate environmental factors such as humidity and temperature. Also the growth phase of the organism of interest has been shown to influence motility. Furthermore, light can influence taxis directly through phototaxis, but also indirectly, by changing parameters such as temperature and humidity. Control experiments for the effect of illumination are therefore best performed on plates wrapped in black cloth. Recently, photoreceptor proteins, and corresponding light responses, have been shown to be present in a wide range of chemotrophic organisms (27). Because in this case the light only serves as an environmental signal and not as a source of energy, as can be the case in phototrophic organisms, this may simplify detailed (mathematical) description of phototaxis responses (28).

As compared to experiments of population responses on an agar surfaces, which is the most extensively applied technique, studies of migration in a glass microscope chamber (29) have the advantage that responses can be recorded in a few minutes, so

that dominant effects of gene expression, in the response toward alteration of the illumination regime, can be excluded.

1.2.2. Swarming Motility in *Rhodocista centenaria*

The purple photosynthetic bacterium *Rhodocista centenaria* (formerly *Rhodospirillum centenum*) can undergo a differentiation from swimming cells to swarming cells. Whereas swimming cells in liquid medium contain only a single polar flagellum, cells grown on solid surfaces develop numerous lateral (peritrichous) flagella in addition to the polar flagellum (30, 31). In liquid culture *R. centenaria* cells use their single flagellum for a light response that is independent of the direction of incoming light, i.e., it is not a phototactic response in the strict meaning of this term (32), but rather very similar to the phototaxis responses in other purple bacteria. However, on solid surfaces, groups of cells of this organism have been shown to exhibit a genuinely phototactic response: cells were shown to exhibit positive phototaxis toward light of long wavelengths (>800 nm), and a negative phototactic response toward light of shorter (<600 nm) wavelengths (30). The molecular signal transduction pathway underlying the swarming response has been elucidated in great detail. *R. centenaria* has three *che* operons, of which two have been shown to be involved in both swimming and swarming cell motility. Where the *che1* operon directly regulates taxis in a manner similar to the *Escherichia coli che* paradigm, the *che2* operon is involved in flagellar biosynthesis (33). Presumably, this response is not based on a dedicated photosensory protein, but rather on sensing of (the rate of) electron flow through the photosynthetic electron transfer chain (34). However, because both wavelengths for positive and for negative responses include wavelengths of bacteriochlorophyll absorption, there must be a more complex regulatory mechanism at the basis of these processes (9). Internal colony light gradients may also play an important role in this light sensing.

1.2.3. Twitching Motility in *Synechocystis*

Also in cyanobacteria, a phototaxis response of cells involved in surface migration has been identified and studied in great detail. *Synechocystis* sp. PCC6803, the first phototrophic organism with a fully sequenced genome (35), is able to migrate through twitching motility. Various types of photoresponses are known to occur in this organism, including both negative- and positive phototaxis. Most published phototaxis experiments in *Synechocystis* are performed using soft agar plates, although also glass slide-based methods have been described (29). On solid surfaces, *Synechocystis* cells can move and form expanding, flat, and irregularly shaped colonies. This type of movement has been shown to be dependent on type IV pili. *Synechocystis* has two, morphologically different types of pili, thick and thin, but only the thick pili, for which the structural gene is *pilA1*, are required for motility (36). In several other bacterial species thin pili have an opposite function, i.e., to attach the cells to a solid surface (37).

To capture a light signal, ultimately resulting in a phototactic response, *Synechocystis* has various possible photoreceptor proteins at its disposal: The presence of the phytochromes Cph1 and Cph2, binding a linear tetrapyrrole, has been relatively long known. In vitro studies have shown that Cph1 absorbs predominantly red light, as most phytochromes, but the absorption of Cph2 is mainly in the blue part of the spectrum (38); the flavin-binding BLUF protein PixD (Slr1694) (39) is a typical blue-light receptor. The third phytochrome-like protein in this organism is PixJ1. Although in vitro experiments have suggested that this is a blue/green photoreversible light receptor (40), in vivo experiments suggest that it has authentic red/far-red photoreversibility (29). Interestingly, PixJ1 in its carboxy-terminal region shows homology to MCPs, whereas the other orfs in this operon (*sll0038–sll0043*) all show homology to Che proteins, involved in flagellar switching in chemotaxis and in type IV pili biogenesis. The best-studied light response in *Synechocystis* is positive phototaxis (41). Disruption of *pixD* abolishes the attraction of the organism by red light; it even results in a repellent effect of light of this color, i.e., from positive to negative phototaxis (39)(Fig. 2), as does deletion of *pixJ1* (41, 42).

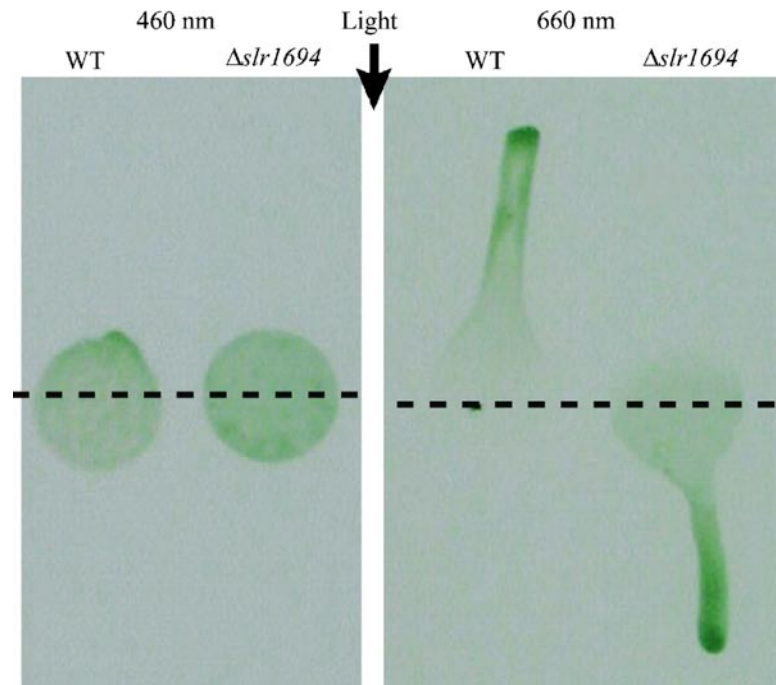


Fig. 2. Phototactic movements of colonies of the *slr1694* gene-disrupted mutant ($\Delta slr1694$) and its parent strain, *Synechocystis* PCC-P. 1 μ l of each cell suspension was spotted and grown for 44 h under lateral illumination (arrow) at 460 nm (left) and 660 nm (right). The dotted line shows the initial position of inoculation before the illumination. Reprinted with permission from ref. 39.

Both the various photoreceptor proteins that provide input signals for the phototaxis signal transduction cascade, and the resulting responses of pili synthesis and retraction that form the output of this phototaxis process have been characterized in some detail. The intermediate signal transduction steps that link the photosensory input signals to changes in pili structure are still less well understood. A tentative working model to unify the majority of the observations available from literature on this system is shown in **Fig. 3**. The signaling cascade starts with photoactivation of PixD, of which the molecular basis has been resolved in high detail: Both yeast two-hybrid screens (39) and biochemical experiments (43) have shown that PixD interacts with the PatA-like response regulator PixE. Light absorption alters the multimeric state of the PixD-PixE complex, resulting in the release of PixE molecules from the complex, presumably enabling these proteins to propagate the signal (43). In a large-scale analysis of protein-protein interaction in *Synechocystis*, glycogen phosphorylase and glucokinase were identified as additional interaction partners of PixD (CyanoBase, <http://beta.kazusa.or.jp:3018/genes/show/slr1694>; see also ref.44), directly linking light activation with glucose catabolism and intermediary metabolism. PixD may modulate the activity of glucokinase and glycogen phosphorylase, with the result that cAMP levels might increase in the light. An effect of light on cAMP levels (which are mostly regulated by the adenylate cyclases Cya1 and Cya2) has been demonstrated (45). Increases in cAMP levels by blue-light stimuli are mediated by Cya1 (46), but Cya1 activity itself is insensitive to light (47). cAMP levels can directly regulate PilA expression via the cyclic AMP-binding protein SYCRP1 (48). Light signaling through cAMP levels and SYCRP1 specifically regulates phase 3 of the response to a light-stimulus, in which cells move rapidly in fingerlike projections (49). Pili expression is primarily governed by the stress sigma factor, σ^F (50). Expression of this group 3 sigma factor is subject to autoregulation, and to activation by cAMP-activated SYCRP1 (48). σ^F recognizes the promoter sequences of *pilA1* (51), and, interestingly, also the promoter of the phytochrome-encoding gene *slr0041* (51). This latter photoreceptor is responsible for the red/far-red photoreversibility of the attractant response of *Synechocystis* toward low and moderate light intensities ((29), see also **Fig. 3**). In view of the similarity between σ^B from *Bacillus subtilis* and σ^F , it is of significant interest to find out which other stress signals, besides salt (52), activate this latter sigma factor.

1.3. Light-Regulated Motility in Nonphototrophic Bacteria

Whereas light responses were long thought to be present only in phototrophic bacteria, recently, more and more light-regulated responses have been observed in chemotrophic bacteria as well (27). Especially because of the wealth of genomic data becoming

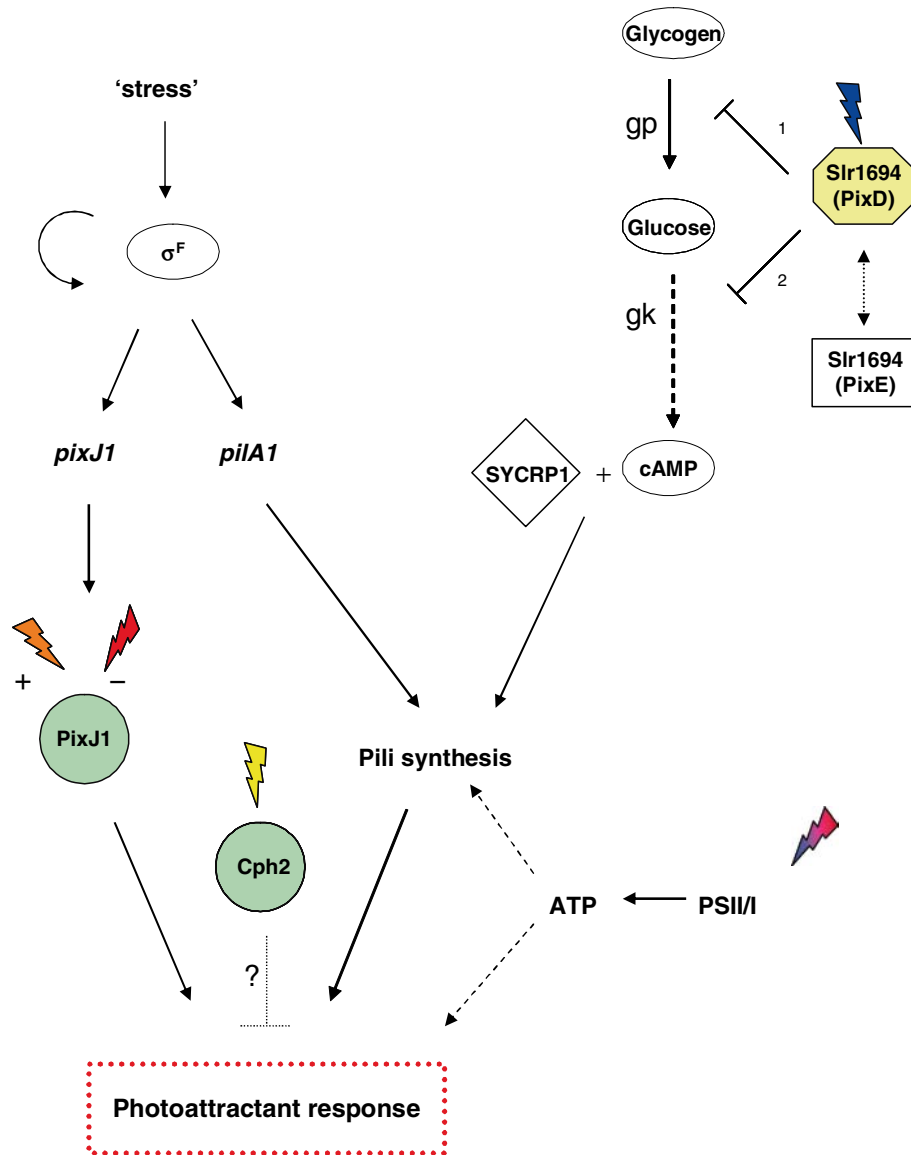


Fig. 3. Working model of regulation of phototaxis in *Synechocystis* sp. PCC6803. See text for detailed description. “1” and “2” indicate interference with the reactions catalyzed by glycogen phosphorylase (gp) and glucokinase (gk), as found by yeast two-hybrid screening methods (44), which is relieved upon blue-light activation of PixD. The circular arrow upstream of σ^F refers to the process of autoactivation to which this sigma factor is subjected. PixJ1 (=TaxD1) is a photoreversible phytochrome (29).

available, and the identification of genes encoding homologs of known photoreceptor proteins in genomes, it is now becoming clear that many chemotrophic bacteria may exhibit previously unsuspected light responses.

The first example is the well-studied bacterium *Escherichia coli*, which encodes in its genome the BLUF-domain containing protein

YcgF (as its only photoreceptor protein). This recently discovered photoreceptor protein binds flavin to yield a blue-light photoreceptor (53) of the BLUF type. *E. coli* serves as the model system for chemotaxis, but until now, no physiological light-regulated motility response has been described in this organism (in contrast to nonphysiological effects of visible light; see e.g., ref. 54). Recently, however, it was shown that light regulates both adhesion on solid surfaces and motility in this organism (Key et al., unpublished results; see also ref. 55). Presumably, the YcgF protein modulates, through signaling-state formation in its BLUF domain, the activity of its output domain, which shows homology to EAL domains. This in turn may affect the level of the signaling molecule bis-(3'-5')-cyclic-diguanosine monophosphate (c-di-GMP) in the cell (56). The observations are most straightforwardly explained by assuming that light-stimulated exopolysaccharide synthesis impairs flagella-based swimming motility.

Acinetobacter sp. ADP1, a common soil bacterium that shows an interesting system for natural transformation (57), does not possess flagella but instead uses polar thick fimbriae for surface migration through twitching motility (58). The organism has thin pili too, but these seem to function in adhesion rather than in migration (M. Bitrian, unpublished observation; compare (37)). Recently, we observed that migration through twitching motility in *Acinetobacter* sp. ADP1 is modulated by illumination with visible light (Bitrian et al., unpublished results); however, the molecular basis of this effect has not yet been resolved. Interestingly, the genome of this organism encodes four photoreceptor proteins of the BLUF-type (27).

A third example was recently published by Oberpichler et al. (7). Light turns out to inhibit motility of *Agrobacterium tumefaciens* (and several other *Rizobiaceae* species) on LB soft agar "swimming plates" via inhibition of the synthesis of the major flagellin subunits FlaA,B. Surprisingly, none of the three tested photosensory proteins encoded in the genome of the organism (i.e., two bacteriophytochromes and a cryptochrome) seems to be involved in this response.

In the following sections, the methods for phototaxis measurements in liquid cultures of *Halobacterium salinarum*, *Rhodobacter sphaeroides*, and *Halorhodospira halophila* will be described in detail.

2. Materials

2.1. Growth of *Halobacterium salinarum*

1. Peptone medium (59): 250 g NaCl, 20 g MgSO₄·7H₂O, 2 g KCl, 0.2 g CaCl₂, and 10 g bacteriological peptone are required per liter.

2. Trace metal solution (1 ml/1 peptone medium): 3 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 23 g FeCl_2 , 4.4 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in 1 l of 0.01 N HCl.
3. A filter apparatus for sterilizing the trace metal solution.
4. Bacto-Agar (Difco).
5. Erlenmeyer flasks.
6. A rotary shaker thermostatted at 38°C (for example a New Brunswick Model G-2 gyrorotary shaker set at 240 rpm).

2.2. *Halobacterium salinarum* Phototaxis Measurements

1. Microscope slides and cover slips.
2. Interference filters in the range from 390 to 650 nm.
3. Infrared-sensitive camera (Newvicon, 1 in., RCA Product Div., Lancaster, PA).
4. Optical shutter (Vincent Associates, Rochester, NY).
5. Infrared transmitting beam splitter (Edmund Scientific, Barrington, NJ).
6. Computerized cell-tracking system (Motion Analysis Systems, Inc., Santa Rosa, CA).

2.3. Growth of *Rhodobacter sphaeroides*

1. 10× concentrated growth medium for *Rb. sphaeroides* (60): 34.8 g K_2HPO_4 , 5.0 g $(\text{NH}_4)_2\text{SO}_4$, 40.0 g succinic acid, 1.0 g L-glutamic acid, 0.4 g L-aspartic acid, 5.0 g NaCl, 2.0 g nitrilotriacetic acid, 3.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.334 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.020 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 ml $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (1% solution), 1 ml trace elements solution, and 1 ml vitamin solution are required per liter.
2. Trace element solution (per 100 ml): 1.765 g EDTA, 10.95 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.54 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.392 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.248 g $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.114 g H_3BO_3 .
3. Vitamin solution (per 100 ml): 1.0 g nicotinic Acid, 0.5 g thiamine HCl, 0.010 g biotin.
4. Casamino acids (Difco Laboratories) and a 1 M KCl solution.
5. A flat glass growth chamber (approx. 2 mm thick) to ensure homogeneous illumination of the bacterial culture.
6. An illuminated incubation area thermostatted at 30°C.

2.4. Surface Tethering of *Rhodobacter sphaeroides*

1. 10 mM HEPES buffer of pH 7, containing 100 µg/ml chloramphenicol to arrest protein synthesis. This solution is used to wash the cells before tethering to a glass surface.
2. Optically flat capillaries (Camlabs, Cambridge, UK).
3. Sigmacote (Sigma-Aldridge, Poole, UK), a hydrophobic agent used to coat the capillaries.
4. Antifilament antibodies to tether the cells to the glass surface of the optically flat capillaries (0.1 × 1 mm, Camlabs, Cambridge, U.K.).

2.5. *Rhodobacter sphaeroides* Phototaxis Measurements

1. A long-bandpass filter (>950 nm) placed in the optical path of the microscope before the bacterial sample. This light is used to observe the bacteria without driving photosynthesis.
2. A quadrant photodiode (PIN-SPOT 4DMI, UDT Sensors, Inc., Hawthorne, CA) to detect rotation of the tethered cells.
3. A 100-W tungsten-halogen light source equipped with a 500–820-nm bandpass filter ($\sim 270 \text{ Wm}^{-2}$ light intensity, measured using a quantum radiometer, e.g., from Li-Cor, Lincoln, Nebraska) to provide photostimuli to the cells that initiate photosynthesis.
4. An optical shutter to allow precise timing of the photostimulation of the cells.
5. A beam splitter to allow the coaxial illumination of the cells with >950 nm observation light and 500–820-nm photostimulation light (Fig. 4).

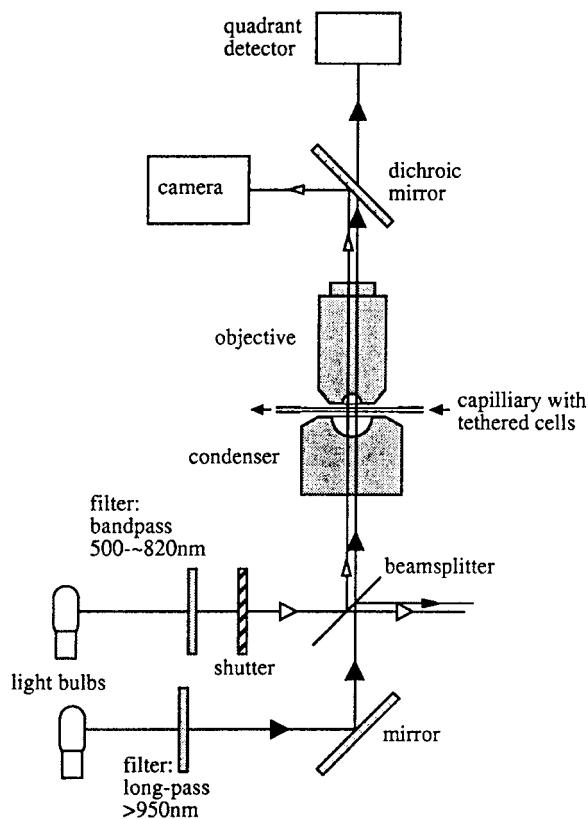


Fig. 4. Experimental setup used for phototaxis measurements on tethered cells of *Rhodospirillum rubrum*. Light for microscopic detection of the cells (>950 nm) and for providing photostimuli to the cells (500–820 nm) are controlled independently. The rotation of individual cells is quantified using a quadrant photodiode. Reprinted with permission from [ref. 18](#).

2.6. Growth of *Halorhodospira halophila*

1. For growth of *H. halophila* (61): 0.8 g KH_2PO_4 , 0.8 g NH_4Cl , 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 g NaAcetate, 200 ml 1 M Na_2CO_3 pH 9.0, 1 g $\text{Na}_2\text{S}_2\text{O}_3$, 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 130 g NaCl, and 1 ml trace solution (see next) are required per liter.
2. Trace solution (per 1 l): 1.8 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.25 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 70 mg $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 g ZnCl_2 , 0.5 g H_2BO_3 , 30 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 10 mg $\text{NaSeO}_3 \cdot 5\text{H}_2\text{O}$.
3. Glass screw-cap tubes or bottles with a rubber ring to ensure anaerobic conditions during growth.
4. Bacto-agar (DIFCO).
5. AnaeroGen Compact transparent pouches (An0010C) from Oxoid (Cambridge, U.K.) with atmospheric generation system for anaerobic growth on agar plates.
6. A thermostatted water bath illuminated by 60-W Tungsten light bulbs (approximately $50\text{--}75 \mu\text{mol photons} \cdot \text{m}^{-2} \text{ s}^{-1}$) for growth of liquid cultures.
7. A thermostatted incubator containing light bulbs for illumination of bacterial agar plates.

2.7. *Halorhodospira halophila* Population Phototaxis Assay

1. Optically flat glass capillaries (see earlier).
2. Broadband optical filters and narrow bandwidth optical interference filters for providing phototaxis stimuli and for microscopic detection of the cells.

2.8. *Halorhodospira halophila* Phototaxis Measurements: Step-Up and -Down Responses and Wavelength Dependence

1. Optically flat glass capillaries.
2. A broadband filter transmitting light above 550 nm, but not in the range 400–500 nm.
3. Narrow bandwidth (9 nm) interference filters in the spectral range 400–500 nm.
4. A Schott KL 1500 halogen lamp (150 W) with an optical fiber.

3. Methods

H. salinarum and *Rb. sphaeroides* can be grown chemotrophically in the dark or photosynthetically in the light. The observed photoreponses will depend critically on the conditions under which these cells are grown. For example, responses triggered by SRI dominate in *H. salinarum* grown under anaerobic photosynthetic conditions, while SRII is the main photoreceptor after aerobic chemotrophic growth. Thus, great care should be taken

in choosing growth conditions for cell cultures to be examined for phototaxis responses.

Phototaxis responses can be observed using a number of distinct assays: (1) the pattern of accumulation into or away from a light spot with a specified spectral composition projected into a bacterial culture (22, 32); (2) the effects of sudden changes in light intensity on the motility response of single cells tethered to a glass surface via their flagella (18, 19); (3) the taxis response of individual free-swimming bacteria in a population of cells after a sudden change in light intensity (22, 62, 63). The wavelength dependence of these responses can yield critical information on the photoreceptor that triggers the observed response.

The microscopic observation of bacteria in phototaxis experiments introduces a possible complication: the light used to observe the cells can generate phototactic signals. Thus, it is critical that the light used to observe the cells microscopically is chosen to be in a spectral range that does not trigger the phototaxis responses under study. An additional complication in the case of positive phototaxis in purple bacteria is that the energy status of the cells needs to be sufficient to allow swimming to occur, and the “photoreceptor” for photosynthesis and phototaxis is the same: the photosynthetic machinery. In the case of *H. salinarum*, the blue-shifted S_{373} intermediate, which is formed by attractant orange light, is the photoreceptor for a negative phototaxis response to near-UV light. Thus, the photoresponse toward near-UV light is only observed when the cells are simultaneously illuminated with light that initiates the photocycle of SRI (64). These “spectral complications” need to be carefully taken into account when designing experiments.

3.1. Phototaxis of *Halobacterium salinarum*

3.1.1. Growth of *Halobacterium salinarum*

1. To prepare the peptone medium, the components are dissolved in distilled water in the order listed, and the pH is adjusted to pH 7.0 with 4 N NaOH, using a pH electrode with a low sodium error.
2. The medium is sterilized by autoclaving and cooled down to room temperature.
3. The trace metal solution is prepared in 0.01 N HCl, filter sterilized, and added to the autoclaved peptone at 1 ml/l peptone medium.
4. The cells are grown aerobically in the dark at 38°C in Erlenmeyer flasks (typically 700 ml in a 2-l flask) using a rotary shaker.
5. For phototaxis assays, the cells are grown to the late exponential phase, diluted ~100-fold in fresh medium, and grown for ~2 h for use in motility measurements. The phototaxis response that will be observed depends critically on the strain of *Halobacterium salinarum* that is used (see Note 1).

6. To maintain the cells, they are grown on slants containing the peptone medium plus 1.5% Bacto-Agar.

3.1.2. *Halobacterium salinarum* Phototaxis Measurements

1. The cells are placed on a microscope slide, covered with a cover slip, and observed with dark field optics using >700 nm (730–850 nm) light using a 100-W tungsten-halogen lamp (62, 65). Images are recorded using an infrared-sensitive camera mounted on the microscope. During the phototaxis measurement, the cells are maintained at 37°C using a water-jacketed and thermostatted slide holder on the microscope stage.
2. The cells are exposed to light stimuli from a 150-W Xe arc lamp, 200-W Hg arc lamp, or 100-W Hg–Xe lamp. The light from these lamps was passed through interference filters in the region 390–650 nm and an electronically controlled optical shutter. The filtered light from these lamps is combined with the infrared monitoring beam using a visible reflecting, infrared transmitting beam splitter. Cells can be exposed to either step-up or step-down responses lasting 3–90 s or light pulses lasting 10–100 ms.
3. The recorded images of swimming cells are analyzed using the EV1000 computerized (66) cell-tracking system to extract the time-dependent reversal frequency of the cells (Fig. 5; see for example ref. 67).

3.2. Phototaxis of *Rhodobacter sphaeroides*

3.2.1. Growth of *Rhodobacter sphaeroides*

1. Add the components of the 10× medium to 850 ml of distilled water while stirring. Bring the volume to 1 l with distilled water. The pH will be in the range 4.5–4.9. The solution can be frozen for later use.
2. Add the components of the trace element solution to 85 ml of distilled water while stirring. Bring the volume to 100 ml with distilled water. Store the solution at 4°C.
3. Add the components of the vitamin solution to 85 ml of distilled water while stirring. Bring the volume to 100 ml with distilled water. Store the solution at 4°C.

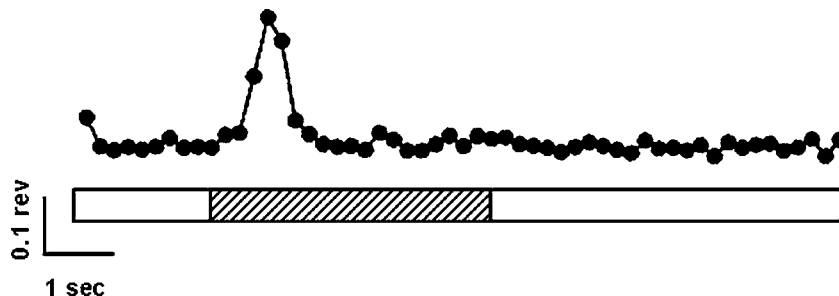


Fig. 5. Detection of a repellent response in *Halobacterium salinarum* to a step-down in orange light intensity. The reversal frequency in a population of cells is determined as a function of time using computerized motion analysis software. The step-down stimulus is indicated by the hatched bar. Reprinted with permission from ref. 67.

4. Add 100 ml of the 10× medium to 800 ml distilled water.
5. Adjust to pH 7.0 with KOH.
6. Add 2 g casamino acids per liter 1× medium (optional).
7. Bring volume to 1 l with distilled water.
8. Sterilize by autoclaving.
9. *Rb. sphaeroides* cells (*see* [Note 2](#)) are grown anaerobically at 30°C in clear and flat glass bottles with airtight caps under bright light illumination (approximately 100 W/m² in the range 400–800 nm).
10. The cells are harvested in early exponential phase at low-to-intermediate cell densities.

3.2.2. Surface Tethering of *Rhodobacter sphaeroides*

1. The optically flat capillaries are coated with Sigmacote.
2. Anaerobic tethering buffer (10 mM Na-HEPES, pH 7.2 containing 100 µg/ml chloramphenicol) is prepared by sparging with nitrogen gas (*see* [Note 3](#)).
3. One milliliter of cell culture is harvested by centrifugation and resuspended in anaerobic tethering buffer. The resuspended cells can be illuminated for up to 1 h to ensure anaerobic conditions.
4. A 5-µl volume of cells is incubated for 30 min at 30°C with 2 µl of polyclonal antibody (135 µg of protein per ml) directed against *Rb. sphaeroides* flagellin ([18](#), [19](#)). This polyclonal antibody was obtained by shearing the cells to break off flagellar filaments. Purified flagellar filaments are used to generate a polyclonal antibody in New Zealand white rabbits. This polyclonal antibody is adsorbed using a nonflagellated strain of *Rb. sphaeroides* to remove antibodies directed against the cell body. The antibody is stored at –15°C.
5. The cell-antibody mixture is then introduced into the coated optically flat capillaries, which are then sealed with Vaseline and incubated for 20 min to allow attachment of the flagella to the glass surface. Since the flagellum is tethered to the glass surface, flagellar rotation will result in the rotation of the cell body with respect to the tethering point on the glass surface. This rotary motion of the cell is measured.

3.2.3. *Rhodobacter* *sphaeroides* Phototaxis Measurements

1. The cells tethered to the surface of the optically flat capillary are observed using a Nikon Optiphot (or similar) microscope with a 100× oil immersion phase contrast objective using light >950 nm to image the cells ([18](#)).
2. This light is projected onto the quadrant photodiode such that the image of the tethered cell is aligned to have the center of cell rotation.
3. The currents from the four quadrants of the photodiode (labeled *a–d*) are sampled at 128 Hz, and used to calculate

the rotation of the bacterium as expressed in terms of X and Y signals calculated as follows:

$$X = ((a + d) - (a + c)) / (a + b + c + d)$$

$$Y = ((a + b) - (c + d)) / (a + b + c + d)$$

4. The signals for a specific time range are selected. The extremes of the signals of X and Y in the data are fitted to envelopes, and these envelopes are subtracted from the data to correct for drift in the signals. The data are scaled to a scale from -1 to 1 .
5. The resulting corrected X and Y signals are used to calculate the speed of rotation of the cells using the following procedure. The angle θ is calculated as $\arctan(Y/X)$, and converted into $\cos \theta$ and $\sin \theta$. The speed of bacterial rotation is then calculated as $1/2\pi (d\theta/dt)$.
6. Just before starting the experiments, the cells are illuminated with light that is absorbed by the photosynthetic machinery.
7. The cells are exposed repeatedly by light from the second light source (in the range 500–820 nm) using an optical shutter.
8. The effects of the illumination of the cell on the rotation speed of individual bacteria as a function of time after opening or closing the optical shutter are determined.

3.3. Phototaxis of *Halorhodospira halophila*

3.3.1. Growth of *Halorhodospira halophila*

1. *H. halophila* cells are grown in solution in the growth medium described by (61). The $MgCl_2$, $CaCl_2$, Na_2S , NH_4Cl , and trace solution are added from autoclaved stock solutions to an autoclaved stock solution of the other components to avoid precipitation of salts. After mixing, the pH is adjusted to 8.5 with a filter-sterilized solution of 3 M HCl. Typically, cells are inoculated in prewarmed medium with 5% volume from a liquid culture in the late-exponential or stationary phase with an OD of ~ 1.5 at 540 nm. The cells are incubated at 41–43°C in a thermostatted water bath, with the well-sealed tubes or bottles submerged $\sim 80\%$ into the water bath, and illuminated with Tungsten light bulbs (see Note 4). Depending on the light intensity, the doubling time can be ~ 10 h.
2. For growth of *H. halophila* on agar plates, at least 0.6% (wt/vol) agar is added to the Imhoff medium prepared as described earlier. After inoculation of the plates with *H. halophila* cells, the agar plates are sealed in AnaeroGen Compact transparent pouches (An0010C) from Oxoid (Cambridge, U.K.). The atmospheric generation system for these pouches is used to generate anaerobic growth conditions. The sealed pouches are incubated at 40–43°C under illumination with a 60-W Tungsten lamp inside the incubator until deep red colonies of *H. halophila* appear.

3.3.2. *Halorhodospira*
halophila Population
Phototaxis Assay

1. *H. halophila* cells from a liquid culture in the late exponential phase with a high percentage of motile cells (*see* [Note 5](#)) are incubated in a glass capillary. The open ends of the capillary are sealed with Vaseline to create an anaerobic environment in the capillary ([22](#)).
2. The cells are exposed to green light for ~30 min that excited photosynthesis to ensure the energy status of the cells.
3. The light of the microscopy (for example a 100-W halogen lamp in a Nikon Optiphot microscope) is passed through an optical filter to provide a light climate of choice, and through a diaphragm to illuminate only a portion of the cell suspension that is viewed using an $\times 125$ phase contrast objective.
4. The cell suspension is exposed to this light climate for ~10 min. The light intensity reaching the bacteria can be measured using a photometer, for example a Unit SKP200 photometer with an SKP215 sensor head (Skye Instruments, Portree, Isle of Skye, Scotland).
5. The diaphragm is then manually rapidly adjusted to homogeneously illuminate the entire area in view, and then bacterial culture is photographed. This procedure can be repeated with various optical filters to document the wavelengths involved in the phototaxis responses. Note that the same spectral composition is used to photostimulate and detect the cells (*see* [Note 6](#)).
6. Phototaxis responses will result in a nonhomogeneous distribution of bacteria in the field of view (**Fig. 6**). Positive phototaxis results in the accumulation of bacteria in the circle that was illuminated during the 10-min incubation period. This is the case for *H. halophila* when green light is projected into the culture ([22](#)). Negative phototaxis results in a reduction in cell density from the illuminated spot. A combination of negative and positive phototaxis, as is the case for *H. halophila* when blue light is used (**Fig. 6**), results in the accumulation of bacteria at the edge of the illuminated light spot ([22](#)).

3.3.3. *Halorhodospira*
halophila Cellular Photo-
taxis Measurements:
Step-Up and -Down
Responses and
Wavelength Dependence

1. *H. halophila* cells from a liquid culture in the late exponential phase are incubated in a glass capillary with its ends sealed with Vaseline to create an anaerobic environment in the capillary. The cells are then exposed to green light for ~30 min ([22](#)).
2. A broadband filter with an absorbance maximum near 450 nm is inserted into the optical path of the microscopy (before the cell sample), and the cells are adapted for ~10 min to these light conditions.
3. To measure the cellular response to a step-up in blue-light intensity, the broadband filter is rapidly removed (manually)

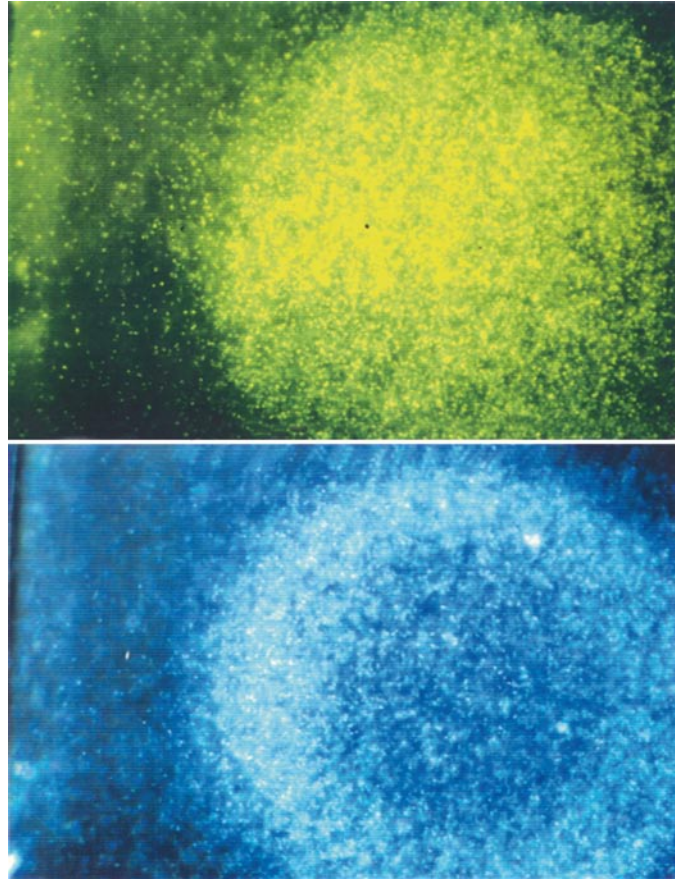


Fig. 6. Population phototaxis assay for *Halorhodospira halophila*. A green-light spot (*top*) causes cells to accumulate in the light due to positive phototaxis. A blue-light spot (*bottom*) triggers both positive phototaxis toward the light and negative phototaxis away from intense blue light, resulting in a *ring-shaped* accumulation pattern. Reprinted with permission from [ref. 22](#).

from the optical path, and the resulting response of the cells is monitored and recorded on a video recorder for subsequent analysis (either manually or preferably using cell-tracking software).

4. To measure the response to a step-down in light intensity, the same broadband filter can be rapidly inserted into the light beam.
5. To measure the wavelength dependence of the step-up response, the cells are detected using light above 550 nm that is saturating for photosynthesis and are exposed to this light for ~10 min before starting the measurement.
6. A Schott KL 1500 halogen lamp (150 W) with an optical fiber is used to illuminate the capillary. Narrow bandwidth interference

filters are used to select photoexcitation wavelengths in the range 400–500 nm. The light intensity transmitted by each filter is determined using a photometer and adjusted to be in the same using neutral density filters. The effect of 4-s exposures of the cells (achieved using an optical shutter and shutter control box, Vincent Associates, Rochester, NY) is determined as described under **step 3**.

3.4. Concluding Remarks

Whereas during the last two decades of the previous century behavioral responses in bacteria were routinely interpreted within the framework of the *E. coli* chemotaxis response, this approach now is no longer tenable. Not only do we know examples of Che-like proteins that are not involved in motility responses, like the Wsp-system of *Pseudomonas aeruginosa* (68) which plays a role in biofilm formation, but in addition, light-modulated effects on bacterial motility have now been documented in which no Che-like proteins seem to play a role (see earlier). For this reason, resolving the molecular basis of these responses has become more challenging. From now on also effects of intermediary metabolism and modulation of gene expression have to be considered as possible underlying mechanisms.

An additional challenge is to find out which of the candidate photosensory proteins plays a role in each of the various responses recently discovered in chemotrophic bacteria. Nevertheless, these latter systems also bring the attraction of using light stimuli to optimally control the biological system under study.

4. Notes

1. A large number of strains of *H. salinarum* are available, containing different combinations of mutations in genes relevant for phototaxis responses (69, 70). Complications due to overlapping photosynthetic responses triggered by bacteriorhodopsin (BR) and halorhodopsin (HR) and phototactic responses triggered by SRI and SRII can be avoided by using strains lacking BR and HR, respectively. In addition, strains are available containing only SRI or SRII, allowing photoreponses triggered by each individual photoreceptor to be studied. Strains that are deficient in retinal biosynthesis can be used for studies on the effects of retinal analogs (added to the medium) on phototaxis responses. Finally, methods for selecting highly motile cells are available and have been used to select highly motile strains of *H. salinarum*.
2. Important differences exist between strains of *Rb. sphaeroides*. Strains RK1 and WS8-N respond differently to blue-light stimuli,

and only *Rb. sphaeroides* RK1 contains a *pyp* gene, but this gene is not involved in the observed blue-light response (63).

3. Since the absence or presence of oxygen will affect the rate of electron transport, the concentration of this gaseous compound can strongly influence the observed phototaxis responses. Thus it is critical to control the oxygen concentration that the cells are exposed to during the phototaxis measurements.
4. The emission spectrum of the lamp being used for photosynthetic growth should be considered. *H. halophila* appears to grow better when using tungsten lamps than fluorescent lamps.
5. Loss of motility during growth of *H. halophila* in the laboratory can present an important challenge to studies of its phototaxis responses. Sprenger et al. (22) solved this problem by selecting a motile strain using the following approach. A cell suspension was concentrated by centrifugation and resuspension in small amount of spent medium. This suspension was mixed with an equal volume of 1.5% low-melting agarose cooled to ~45°C. This mix was placed in a hole (removed from the plate using a flame-sterilized metal spoon) in the center of a 0.6% (wt/vol) agar plate with Imhoff's medium and 2 mM Na₂S·9H₂O. The plates were incubated upside down under anaerobic conditions at 40°C for 3 weeks. Deeply purple-colored swarming cells extending from center plug are then cut from the plate using a flame-sterilized knife and diluted into fresh medium.
6. This can represent a problem if obligately phototrophic cells such as *H. halophila* are studied, and light is used that is not absorbed by the photosynthetic machinery. A dedicated photosensory protein may trigger a phototaxis response to this light, but the low-energy status of the cells in this light may preclude the detection of this response. In this case, side illumination of the capillary using an additional light source equipped with a light guide can be used to provide photosynthetically active light. This approach can also be used to separate positive and negative phototaxis responses to distinct colors of light.

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Chapter 3

Photoorientation in Photosynthetic Flagellates

Donat-Peter Häder and Michael Lebert

Summary

Motile microorganisms react to a host of external stimuli, including light, gravity, the magnetic field of the Earth as well as thermal and chemical gradients, in their habitat in order to select a niche suitable for survival and reproduction. Several forms of light-induced behavior have been described in microorganisms including phototaxis, photophobic responses, and photokinesis. Other functions of photoreceptors are regulation of development and entrainment of circadian rhythms. Basically five types of photoreceptor molecules have been identified in microorganisms: BLUF proteins, cryptochromes, phototropins, phytochromes, and rhodopsins. The photoreceptors can control light-activated ion channels or activated enzymes. The responses to the different stimuli in their habitat can be connected in a complex network of signal transduction chains.

Key words: Photoreceptors, Flagellates, *Euglena*, *Chlamydomonas*, Phototaxis, Photokinesis, photophobic responses

1. Introduction

Motile microorganisms respond to a multitude of stimuli in their environment to select favorable habitats suitable for growth and survival as well as reproduction. These intracellular sensory transduction chains triggered by the receptors are linked intracellularly, resulting in a vectorial addition of the response paths. In other cases, the reaction to one stimulus may override that to others; e.g., the reaction to strong light may override gravitaxis to avoid detrimental radiation at the water surface.

Responses to light and gravity are the most prominent reactions in many motile microorganisms ([1](#)). Other environmental clues include chemical gradients such as oxygen and carbon

dioxide (2), availability of food or repellents (3), or pheromones (4). Many organisms are guided by the magnetic field lines of the Earth (5), mechanical (6) or thermal signals and even by electrical fields.

In this chapter we summarize the light-induced behavior of several photosynthetic flagellates with emphasis on the unicellular *Euglena* and *Chlamydomonas*.

2. Photoorientation

Light is one of the major signals for flagellates to orient in their habitat. While being obviously necessary for photosynthetic organisms, also heterotrophic organisms use this stimulus for habitat selection. As described in the following sections, a number of different responses can be distinguished.

2.1. Photokinesis

Photokinesis is a light-induced reaction found in many prokaryotic and eukaryotic organisms (7). It describes the dependence of the movement velocity on the ambient irradiance (8) and is independent of the direction of light. Photokinesis is found in many eukaryotic microorganisms (9) and even occurs in heterotrophic organisms such as the colorless *Astasia longa* (10). In the flagellate *Euglena gracilis* photokinesis is saturated at about 300 lx in white light (11). The photoreceptor for this reaction has not yet been identified but it seems to be a blue light response (12).

2.2. Photophobic Responses or Photoshock Response

Engelmann watched photosynthetic bacteria under the microscope which reversed their swimming direction when they left the irradiated field and entered a dark field as if frightened (13). The older literature is summarized by (14). Cells can be collected in a light field: when the cells enter a light field from the surrounding dark area there is no response, but when they try to leave it they reverse their swimming direction.

This response can also be induced by a temporal change in light intensity: organisms undergo a phobic response when the light intensity suddenly increases or decreases. The same organisms may respond to a decrease in light intensity (step-down phobic reaction) or an increase (step-up response) (15). Phenotypically the reaction can be a sudden stop, a reversal of swimming, or a tumble (16).

In *Euglena*, both step-down and step-up photophobic reactions occur at different irradiances, separated by an indifferent irradiance range with no evident reactions (12). The action spectrum for the step-down photophobic responses is characteristic of a flavin photoreceptor (17) which has recently been

identified as a photoactivated adenylyl cyclase (see below) which belongs to the new family of BLUF proteins (18).

2.3. Phototaxis

Phototaxis describes an oriented movement guided by the direction of light. Organisms swim toward the light source (positive phototaxis) or away from it (negative phototaxis). In many species, spectral sensitivity is highest in the ultraviolet-blue-green spectral region (300–550 nm). Phototactic orientation may be conceptionally easy to be understood, but it involves more than a photoreceptor since it requires an apparatus to determine the vectorial component of light. Some ciliates have developed complicated cellular structures for this purpose which resemble eyes in invertebrates with a lens and a photosensitive layer of pigments even though they are unicellular (19). Directionality of light can be detected with a “one instant mechanism,” where two (or more) photoreceptor(s) measure light in different directions, or a “two-instant mechanism,” where one photoreceptor monitors the irradiance over time usually rotating around its long axis while swimming forward (20).

The photoreceptor molecules used by different microorganisms for light perception vary significantly and fall in different classes including BLUF proteins, cryptochromes, phototropins, phytochromes, and rhodopsins. Other prokaryotic and eukaryotic organisms use photoactive yellow proteins (PYP) which contain a 4-hydroxycinnamate chromophore (21), chlorophylls, carotenoids, phycobilins, and pterins. Hypericins have been found to be involved in photoorientation of ciliates (22).

3. Phototaxis in *Chlamydomonas*

Chlamydomonas reinhardtii is a unicellular, biflagellated, photosynthetic green alga. The cells are ball shaped with a diameter of ca. 10 μm . Located approximately at the equator of the cell is a prominent orange spot with an diameter of ca. 1 μm , the stigma or eyespot. According to the position of the stigma the flagella are labeled *cis* or *trans* flagellum. The flagellar pair typically works in a breast-swimming like fashion for forward motion. Due to the flagellar beating the cell rotates at the same time counter-clockwise. An intriguing advantage of *Chlamydomonas* is the availability of the complete genome sequence as well as efficient transformation systems. In recent years, *Chlamydomonas* became a model system not only for photoorientation and photosynthesis but also for medical research (23).

In *Chlamydomonas* two main photoreactions are observed: phototaxis and photophobic (“photoshock”) reactions. Both

step-up and step-down photophobic responses can be detected (24). The reactions range from a small change in the direction of movement (“threshold response”) to a full stop of the forward movement, a short period of backward swimming (for about one body length), and a resume of the forward swimming in a new direction (25). Two decades ago Hegemann and Marwan determined in a statistical approach that *Chlamydomonas* can detect even single photons (26). Both negative and positive phototaxis are observed in *Chlamydomonas* (27). Foster and Smyth (28) revised an action spectrum for phototaxis in *Chlamydomonas* (29). For theoretical reasons they proposed to use a so-called threshold spectrum in order to circumvent the problems arising from a conventional way of action spectroscopy. In a first approximation an action spectrum should only represent the absorption properties of the photoreceptor pigment. In reality other substances absorbing in the same wavelength range (screen) as the photoreceptor pigment will distort the resulting action spectrum. The revised action spectrum clearly resembled the absorption spectrum of a rhodopsin, i.e., a retinal-binding protein even when at that time no retinal was identified in *Chlamydomonas*. However, Foster and Smyth calculated that per cell only 27,000 retinal molecules were to be expected which made a chemical identification difficult (28). Four years later in fact retinal was found to be involved in photoresponses in *Chlamydomonas*. Phototaxis could be restored in a blind mutant (blocked in carotenoid synthesis) by incubation in retinal analogous (30). The maxima of the action spectra measured after incubation in the retinal analogous changed according to the absorption properties of analogous used. Both phototaxis and phobic responses were restored in these mutant cells by the addition of all-*trans* retinal. Mono-*cis* isomers were less effective (31, 32). Judging from a number of reconstitution experiments with different chromophores suggests that the *Chlamydomonas* photoreceptor contains an all-*trans*, 6-*S-trans* retinal chromophore similar to bacterial rhodopsins. In contrast, animal rhodopsins contain 11-*cis* retinal or the derivatives 11-*cis*-3-hydroxy; 11-*cis*-4-hydroxy; or 11-*cis* 3,4-dehydroretinal. The chromophore undergoes a 13-*trans*- to *cis*-isomerization upon excitation, probably resulting in a conformational change of the protein via the 13-methyl group. The chromophore is accessible by hydroxylamine, which bleaches the chromophore and consequently inhibits phototaxis. After the removal of hydroxylamine, retinal restored the chromophore and the photoresponse reappears (33). It is interesting to note that the β -ionon ring is not essential for photoperception, since a chromophore with only three conjugated double bonds plus methyl groups also restores photosensitivity (31, 32). In light-grown cells, small amounts of 13-*cis* and 11-*cis* isomers were found besides all-*trans*-retinal (34, 35). Kreimer isolated all-*trans* and 11-*cis* retinal from the

chlorophycean alga *Spermatozopsis similis* confirming the results on *Chlamydomonas* (36).

The photoreceptive structure of *Chlamydomonas* is located in and above the stigma, a dark orange array of carotenoid-stained lipid droplets which are arranged in a rigidly packed, hexagonal pattern and often stacked in layers parallel to the cell surface inside the chloroplast, placed on the equator of the cell (37, 38). These structures can be found in many other Chlorophyceae which are capable of orienting with respect to the impinging light. The direction detection is mediated by light reflection and constructive interference in a so called quarter wave stack (39, 40). The layers reflect the light, and the reflected waves interfere with the incoming waves, provided that $\lambda/2$ coincides with the distance between the layers (Fig. 1a). With light impinging perpendicular to the cell perimeter, the resulting maximum appears at the plasmalemma of the cell, where an array of large protein particles has been found, assumed to be the site or associated with the photoreceptor pigments (37, 39). Figure 1b shows a differential interference image of *Tetraselmis chuii*. The efficiency of reflection and interference improves with the number of layers, but only

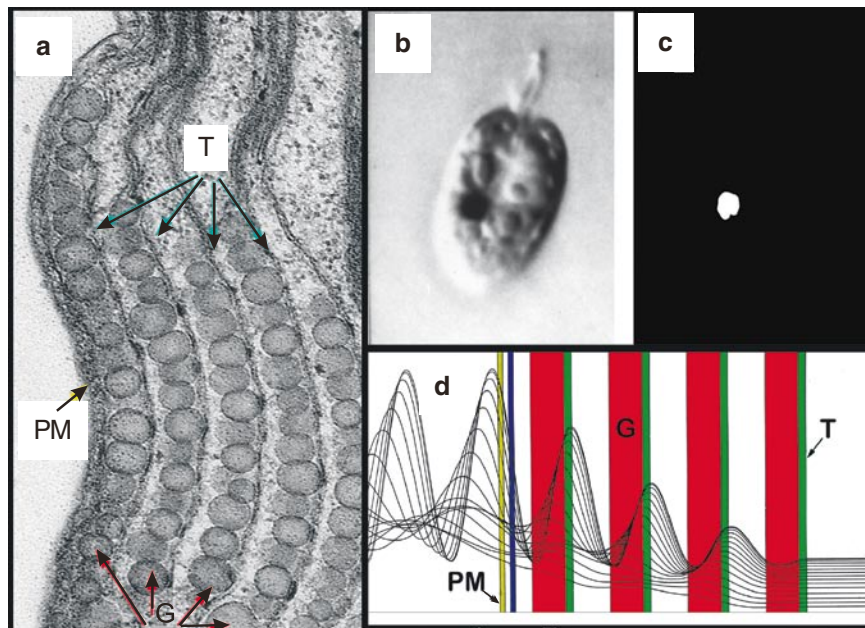


Fig. 1. Schematic representation of the quarter wavelength mechanism for light direction detection in flagellates. (a) Cross section of the stigma of *Hafniomonas reticulata* with several layers of carotenoid-stained lipid globuli (G) with interspersed thylakoids (T). (b) Differential interference contrast image of *Tetraselmis chuii* with the stigma (black dot) and (c) reflection image of the same cell. (d) A laterally incoming light wave is partially reflected at interfaces between layers with high and low refractive indices, spaced at $\lambda/2$. The reflected wave undergoes constructive interference with the incoming wave forming a maximum at the cytoplasmic membrane (PM) overlaying the stigma, the postulated site for the photoreceptor. (reprinted from Kreimer, 2001 (59), with permission).

one layer is sufficient to provide directional information. Light impinging from the rear of the stack is effectively shielded by the chloroplast located toward the cell center (41). Furthermore, the light from that direction would be focused in a region which contains no photoreceptor molecules. The reflective properties of the stigma can be impressively observed by epifluorescence (Fig. 1c). Maximal wavelength sensitivity is determined by the distance between the reflective layers and changes with the angle of the impinging light (42, 43). Threshold phototaxis action spectra have maxima between 460 and 560 nm and are interpreted to be due to the action of rhodopsins. Action spectra for phobic responses peak between 490 and 520 nm (44, 45).

Current approaches for a more in-depth analysis of the photoreceptive structure are centered around proteomics. Until 2005 only a few proteins in the photoreceptive structure were known. These included EYE2 and MIN1 which are both important for the eyespot assembly (Roberts et al., 2001), two splicing variants of a retinal-binding protein (COP) and two 7-helix proteins, the supposed photoreceptors (COP3 and COP4 (46–51)). The proteomic approach revealed a wealth of additional information on the protein composition of the eyespot (52). Newly identified were 202 proteins including calcium sensing and binding proteins, channels, structural proteins as well as enzymes involved in retinal, carotenoid, and chlorophyll biosynthesis. In addition, five protein kinases and two phosphatases were identified. A knock-down of one of the kinases (CK1) resulted in a drastic impairment of hatching, flagellum formation, and circadian control of phototaxis. While the full significance of these findings has to be shown, the occurrence of enzymes involved in phosphorylation makes the close evolutionary relation between the eyespot and animal vision even more intriguing. In animal vision adaptional process are highly dependent on phosphorylation of the rhodopsin. The relevance of the finding of kinases and phosphatases in the eyespot was further emphasized by the identification of three (channelrhodopsin-1) and one (channelrhodopsin-2) phosphorylation sites (53) which might be involved in adaption.

As the cell rotates counterclockwise with a frequency of 1–2 Hz during forward locomotion the perceptive apparatus and the stigma scans the environment for accordingly changing irradiance levels. The photoreceptor molecules (i.e., protein and chromophore) perceive a modulated light signal which ultimately controls the flagellar beat frequency and direction until the cell is aligned with the incoming light rays allowing a net motion either toward (positive phototaxis) or away (negative phototaxis) from the light source. In *Chlamydomonas* the stigma is positioned on the cell equator $\sim 30^\circ$ out of the plane of the two flagella. As a consequence, the maximal light perception occurs a fraction of a second earlier than the reorientation of the flagella, a time interval

which agrees with the time needed for signal transduction from the photoreceptor to the flagella. The stigma is connected to the microtubular rootlet (MTR) that arises from the basal body of the *cis*-flagellum and extends in the direction of the distal end under the plasmalemma (37). Step-up light stimuli induce a short increase in the beat frequency of the *cis*-flagellum and a decrease of the beat frequency of the *trans*-flagellum. A step-down stimulus causes the opposite behavior (54). In demembranated, reactivated *Chlamydomonas cis*- and *trans*-axonemes different Ca^{2+} sensitivities were found (55). Differential motor responses are therefore assumed to be the basis for phototaxis.

Using [^3H]retinal at a concentration just sufficient to fully reconstitute phototactic activity, a 30-kDa protein was found as the only labeled retinal protein in the membrane fraction (56). By treating cytoplasmic membranes with detergent, the photoreceptor retinal was exchanged for [^3H]retinal and the labeled opsin visualized by fluorography (46). The labeled protein was dubbed chlamyopsin, and polyclonal antibodies were used to localize the protein in fixed and permeabilized cells (46). It was found in a spot of 1 μm in diameter at a position where in living cells the eyespot is located. This was also observed in retinal-deficient cells and in stigma preparations of the green alga *Spermatozopsis* (57). In the latter organism the stigma has a light-regulated GTPase activity which is suppressed by addition of the antibodies against chlamyopsin (57–59). The complete chlamyopsin gene (*cop*) was sequenced from an EMBL3 clone (60, 61). In this gene, introns are especially variable in size ranging between 63 and 955 bp. The coding regions of the gene show strong codon biases, with preferentially G or C at the third position (61, 62). The protein is about 65% identical to a similar one found in the colonial alga *Volvox*. Both proteins are highly charged, and transmembrane segments are not obvious; there are only 2–4 segments that are long enough to define transmembrane helices. These retinal-binding proteins show sequence homology to the corresponding animal proteins but not to archaean rhodopsins. In 2001 Fuhrmann and coworkers could show that the until then proposed photoreceptor proteins for phototaxis and photophobic responses are not directly involved in photoperception in *Chlamydomonas* (47). Instead, three groups identified independently two sequences in the *Chlamydomonas* cDNA database which closely resembled microbial rhodopsins. Due to the fact that all three groups submitted the sequences in close proximity a big mix-up in naming conventions had happened. The two proteins were named either channelrhodopsin-1 and channelrhodopsin-2 (ChR1 and ChR2) or *Chlamydomonas* sensory rhodopsin A and B (CSRA and CSRB) or archaean-related sensory rhodopsin 1 and 2 (ASR1 and ASR2). In the *Chlamydomonas* database both genes are named Chlamyopsin 3 and 4 (COP3 and COP4). Applying an RNAi approach

Sineshchekov and coworkers could show the direct involvement of both proteins in photoperception (50, 63). Action spectroscopy with knock-down mutants revealed a maximum sensitivity for ChR1 at 500 and 470 nm for ChR2. Photophobic responses were impaired by reduction in ChR content while the involvement in phototaxis is still unclear. *Chlamydomonas* rhodopsins are light-gated ion channels. While the highest conductance is for H⁺-ions the conductance for calcium plays the dominant role under physiological conditions.

Light activation results in a surprisingly fast change of the electrical membrane potential in *Chlamydomonas* (64, 65). In this organism intracellular recording of the potential changes by micropipettes is difficult due to its small size. The light-induced potential changes can be monitored either by a population method which is based on the asymmetric localization of the signal sources within the cell (66) or by whole-cell recording by means of suction pipettes (64, 67). The latter measurements were performed with cell-wall-deficient strains. The first electrical event after a light flash is an inward current across the portion of the plasma membrane overlaying the stigma. This current has been named “photoreceptor current” (PC) (67, 68). The PC has a $\tau < 20 \mu\text{s}$ which is surprisingly fast. There is a very good correlation between the irradiance and the peak amplitude. There is also a correlation between the delay time (between stimulus and PC) and the irradiance which is as low as 5 μs for high irradiances. The action spectrum for the induction of the PC correlates well with the action spectra of photophobic responses and phototaxis.

The PC is actually the sum of two processes: a fast component, which saturates only at extremely high irradiance levels; and a slow component (69). The maximal amplitude amounts only to 10% of the fast component. The fast potential depends solely on the photoconversion rate of the photoreceptor and is the result of a localized calcium influx (67, 68). The late PC is driven by the transport of about 10^7 elementary charges across the membrane triggered by the absorption of approximately 10^3 photons. This means there is an amplification of about 10,000 (61). The amplification could be due to the activation of GTP: in animal vision one excited rhodopsin can activate up to 500 G proteins, which in turn activate thousands of phosphodiesterase molecules. In *Spermatozopsis* evidence for the involvement of G-proteins in photoperception was presented (57, 58, 70). Light-dependent GTPase activity in isolated eyespot apparatuses was found with an action spectrum similar to that of rhodopsin absorption.

When the sum of the light stimuli exceeds a certain threshold an “all-or-none” response is triggered. The PC becomes superimposed by an action potential-like response (67, 68), which is related to the flagella membrane current and was therefore named “flagellar current” (FC). The FC is due to a massive calcium influx

and is assumed to be driven by voltage-gated Ca^{2+} -channels in the flagellar membrane (68, 71). The FC controls the flagellar-beating pattern and reverses the normal, breast-stroke to the so-called undulation which results in backward swimming of the cell (72). The “fast flagellar current” (F_f) is followed by a “slow flagellar current” (F_s) which seems to be important for the kinetics of backward swimming (64, 72). Subsequent to the FC a K^+ efflux polarizes the membrane again and restores light responsiveness (73–75).

4. Phototaxis in *Euglena*

The unicellular flagellate *Euglena* belongs to the group of Euglenophytes. Locomotion is based on a trailing flagellum which emerges from a basal body inside the reservoir, an invagination at the anterior part of the cell. A second flagellum does not leave the reservoir, but its tip is glued to the emerging flagellum near the paraxonemal body (see later).

In addition to photokinesis, step-up and step-down photophobic responses, *Euglena* shows positive phototaxis at low fluence rates (below 10 W m^{-2}) and negative phototaxis at higher. In contrast to an older hypothesis which assumed the photoreceptor to be located in the stigma, it could be shown to be located in the paraxonemal body (PAB, formerly called paraflagellar body, PFB), seen as a swelling of the emerging flagellum inside the reservoir. The paraxonemal rod (PAR), which lines the whole length of the flagellum, may or may not be involved in light-mediated responses (76).

An earlier hypothesis posited that the light direction is detected by the periodic shading of the PAB by the stigma, located in the cytoplasm next to the PAB outside of the reservoir (77). However, closer investigations indicated that this “shading hypothesis” cannot be the basis for light direction detection: when a population is irradiated with low light intensities from two opposite sides the population splits into two; half of the cells move toward one light source, the other half toward the other (78). At higher irradiances, however, the cells move on the resultant of the vectorial addition of both light directions. Stigmaless mutants of *Euglena* show clear phototactic orientation indicating that the shading hypothesis cannot be correct (79). A final argument against the shading hypothesis results from inhibitor studies: If phototaxis in *Euglena* is based on repetitive photophobic responses, as some investigators posit, inhibitors of the photophobic responses should also impair phototaxis. This could not be verified (78).

In contrast to the shading hypothesis, light direction detection in *Euglena* was found to be based on a dichroic orientation of the photoreceptor pigments (80). This was already suggested by the paracrystalline structure of the PAB (76). The cells show a pronounced polarotaxis and swim at a fixed angle with respect to the e-vector of polarized light (81). During each rotation two positions with a maximal absorption probability occur in lateral light. The role of the stigma seems to be to suppress one of the two maxima by shading. Negative phototaxis additionally requires the screening of the PAB by the rear end of the cell with its chloroplasts facing toward the light source. Chloroplast-free mutants cannot easily distinguish light coming from the front or rear and the cell population splits up into two components showing positive and negative phototaxis, respectively, over a wide range of light intensities.

Several action spectra have been constructed for phototaxis (and photophobic responses) in *Euglena*. They are distinctly different from those measured, e.g., in *Chlamydomonas* and have been suggested to represent the involvement of flavins and pterins. Fluorometric analysis has indicated that pterins, absorbing in the UV-A range of the spectrum with a maximum near 360 nm, function as antenna pigments. Pterins emit at about 450 nm, which corresponds with one of the maxima for flavin absorption. The fluorescence emission of the flavins can be detected at 520 nm (82). Therefore the final photoreceptor is thought to be a flavin.

In another approach, it was speculated that *Euglena* uses a rhodopsin-type photoreceptor (83). Recently, this controversy was finally solved by the fundamental genetic analysis of the receptor. Iseki and coworkers succeeded in identifying and characterizing two related genes involved in light perception (18). The first gene codes for a protein subunit with a molecular weight of 105 kDa and the second for a related one with 90 kDa. Both protein subunits have tandem repeats of a flavin (FAD)-binding motif followed by an adenylyl cyclase activity (Fig. 2). These two subunits have been dubbed PAC α and PAC β ; PAC is an acronym for photoactivated adenylyl cyclase. In the cell the photoreceptor complex consists of two PAC α and PAC β subunits.

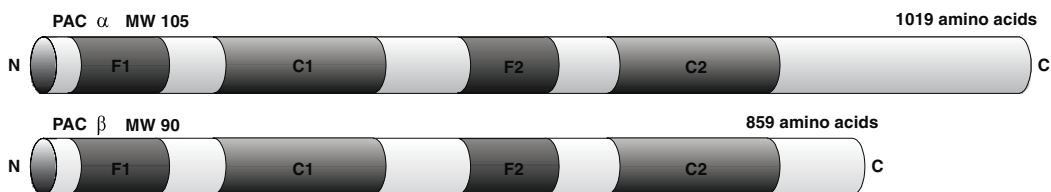


Fig. 2. PAC genes coding for the photoreceptor in *Euglena gracilis*.

This photoreceptor assembly was found to be responsible for the step-up photophobic response, but interestingly not for the step-down photophobic response, which consequently must be mediated by a different photoreceptor.

PCR with primers for the two related genes indicated that both PAC α and PAC β were expressed in a number of phototaxis mutant strains, indicating that these are not primary photoreceptor mutants, but downstream mutants (unpublished data). None of the two proteins was detected in *Astasia*, a non-photosynthetic relative of *Euglena*, which lacks the PAB and consequently does not show phototaxis. Polyclonal antibodies were raised against these two proteins subunits and found to bind at the site of the PAB in the cell, indicating that these represent the photoreceptor.

The final proof was reached by the new and very powerful RNA inhibition (RNAi) technique (84). The technique is based on the fact that double-stranded mRNA is introduced into the cell by, e.g., electroporation. The cells recognize the injected RNA, as a viral invader, reduces it to shorter segments (20–23 base pairs) and disables protein biosynthesis from all similar mRNA templates. Introducing double-stranded mRNA which corresponds to either PAC α or PAC β inhibits the formation of the PAB as shown in interference contrast and fluorescence microscopy. At the same time, it blocked the step-up photophobic response (18).

Further studies showed that the same procedure also blocked both positive and negative phototaxis, but not step-down photophobic responses (unpublished results), indicating that the latter is controlled by a different photoreceptor. Interestingly, higher plants also use photoreceptors, phototropins, with a flavin as a chromophoric group (85).

The signal transduction chain of phototactic orientation has not yet been revealed in detail in *Euglena*. One could speculate that the chromophore is linked *via* a cytochrome to the signal transduction chain, but no evidence is available up to now (86, 87). Some researchers also suggested the involvement of membrane potential changes in signal transduction (88) like in some other flagellates including *Chlamydomonas* (64, 89). However, all attempts to measure a membrane potential by intracellular electrodes have failed up to now. Whole-cell and population methods have also not yet been successful. But the application of the lipophilic cation TPMP⁺, which decreases the membrane potential by entering the cell following the existing potential gradient, moved the threshold light intensity between positive and negative phototaxis to lower irradiances which indicates that the membrane potential is involved in phototaxis. Changes in the membrane potential in *Euglena* are also involved in gravitaxis (90, 91)

The recent results on the genetic analysis described earlier indicate a different linkage to the subsequent steps in the sensory transduction chain. The adenylyl cyclase activity, which has been shown to be light induced by the flavin chromophores on the same photoreceptor molecule, suggests that cAMP is involved in the signaling cascade. This is of specific interest since the involvement of cAMP has been shown in gravitaxis (92, 93). This indicates that the two sensory pathways may converge at the level of this nucleotide. Gravitaxis research also suggests that cAMP could activate a protein kinase which might be speculated to modify axonemal proteins which in turn cause the flagellum reorientation.

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Chapter 4

***Dictyostelium* Slug Phototaxis**

Sarah J. Annesley and Paul R. Fisher

Summary

Dictyostelium slugs are able to respond to environmental stimuli in an extremely sensitive and efficient way. This enables a slug to migrate to more favourable locations for formation of fruiting bodies and dispersal of spores. Phototaxis is a readily assayed phenotype and reflects the interactions of environmental stimuli with morphogenetic signalling systems controlling the movement of the slug. The methods for assaying phototaxis are described here. Qualitative phototaxis tests are described and can be used for rapid screening of potential mutants or effects of pharmacological agents. These tests are simple to conduct yet care must be taken in order to avoid the effects of high cell density which can be misleading when interpreting results. Quantitative phototaxis tests can be performed with known cell densities of amoebae which ensures that any effects seen are caused by the mutation or pharmacological agent and not simply due to differences in cell densities.

Key words: *Dictyostelium*, Phototaxis

1. Introduction

The aggregation of starving amoebae leads to the formation of a multicellular organism – the slug. The slug displays extremely sensitive and accurate orientation behaviours towards physical (temperature gradients and light) and chemical stimuli (1). In the soil environment this enables the slug to migrate to the surface of the soil which is the optimal location for formation of a fruiting body and dispersal of the spores. The surface is the optimal location as it generally contains abundant bacterial food supplies. To migrate to the surface, the slugs use a photosensory apparatus whose sensitivity approaches that of the human retina (responding to energy fluxes as little as $1\mu\text{W}/\text{cm}^2$) (2). Slug phototaxis has been studied for many decades beginning with the

seminal studies by Raper in 1940 (3), Bonner et al. in 1950 (4) and Francis in 1964 (5).

D. discoideum slugs move towards the light bidirectionally, with two preferred directions either side of the light source (6). This phenomenon is explained by a lens effect which was first shown by Francis (5) in combination with a switch between turning away from the light to turning towards the light as the current deviation from the light source direction increases (6). When light is focused onto the convex surface of the slug the light is refracted onto its distal side causing the slug to turn away from the light. This movement creates greater differences in light intensity between opposite sides of the slug as it turns away from the light source. When the slugs have deviated too far from the direction towards the light source they correct this deviation by turning towards the light. This switch between turning away from and turning towards the light produces the bidirectionality observed in slugs and the angle of deviation from the light source at which it occurs is the preferred direction of migration. In wild-type slugs under most conditions the preferred angle of deviation is sufficiently small that slug phototaxis appears unidirectional. However, under specific conditions such as high cell density or in phototaxis mutants this angle becomes greater and bidirectional phototaxis becomes more evident.

Turns towards the light are mediated by light-induced production of a Slug Turning Factor (STF) which is thought to act as a chemical repellent (7). The light focused onto the distal side of the slug stimulates the production of higher concentrations of STF on this side. This difference in STF concentration creates a lateral STF gradient throughout the slug. The slug is able to measure these gradients and turn toward the light source (7).

In addition to STF, there is pharmacological, genetic and/or biochemical evidence for the roles of many components involved in the phototactic signal transduction pathway (1) including G proteins (8), the second messengers cAMP (9), cGMP (10), IP₃ (8) and Ca²⁺ (11) as well as signalling proteins such as RasD (12) GefE and GefL (13), protein kinases such as PKB and ErkB (14) and cytoskeletal proteins such as GRP125 (15), villidin (16), CAP (17), filamin (18) and FIP (19) (Fig. 1). A number of the proteins involved form a photosensory signalling complex that is assembled on the scaffolding protein filamin (14).

Figure 1 indicates that the turning behaviour of the slug is controlled by the slug tip. Light intensities across the slug tip cause lateral shifts in tip position by altering the balance between tip autoactivation and autoinhibition. The shifts in tip position are accompanied by slug turning as the slug follows its tip. Tip activation signals are carried by extracellular cyclic 3', 5'-adenosine monophosphate (cAMP) waves, whereas tip inhibition signals may be borne by one or more of the secreted molecules

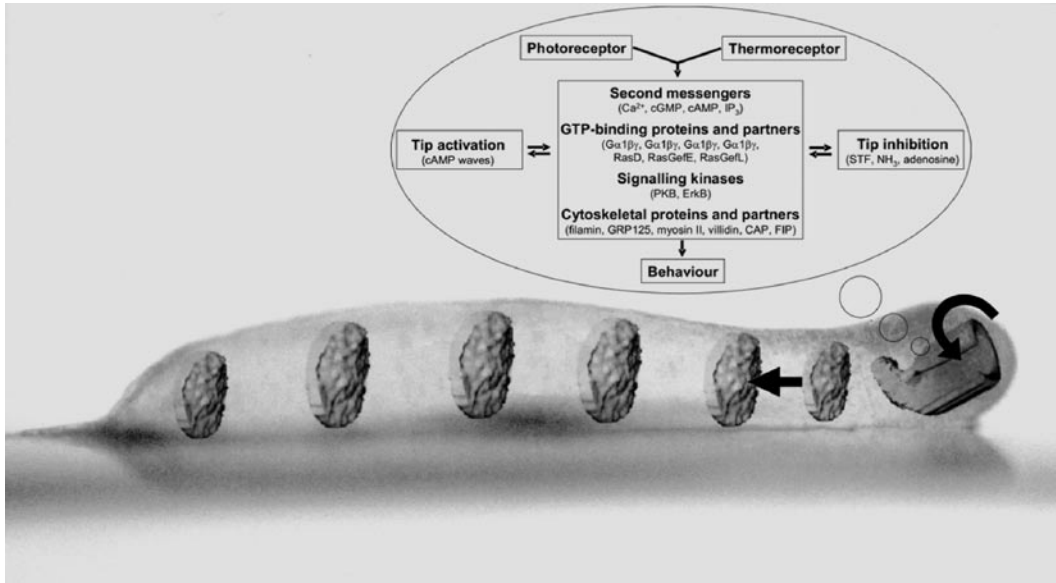


Fig. 1. Signalling pathways controlling slug phototaxis and thermotaxis. A lateral view of a slug migrating on a water agar surface is shown. The 'thought bubbles' emanating from the tip indicate that the tip controls slug behaviour via the indicated pathways. Signals from photoreceptor and thermoreceptor converge early and thence control the concentrations of the intracellular second messengers cyclic 3', 5'-adenosine monophosphate (cAMP), cyclic 3', 5'-guanosine monophosphate (cGMP), Ca^{2+} and possibly inositol triphosphate (IP_3). The evidence for IP_3 involvement is the pharmacological effect of Li^+ , whose target could also (or instead) be glycogen synthase kinase 3 (GSK3). Heterotrimeric G proteins, the small guanosine triphosphate-binding protein RasD and other downstream signalling proteins are involved in transducing the signals. These in turn modulate the tip activation and inhibition signals that determine the position of the slug tip. Transient lateral imbalances between tip activation and inhibition induced by this means by light and temperature gradients cause temporary lateral shifts in tip position and thence slug turning because the slug 'follows its nose'. Depending on whether tip activation or inhibition dominates the response, the slug turns either towards or away from the light source. Sign reversals in slug turning responses result from switches in the balance between control by tip activation and inhibition. This explains direction-dependent sign reversals in phototaxis that cause bidirectional phototaxis (see text). Tip activation signals are believed to be carried by three-dimensional spiral scroll waves of extracellular cAMP analogous to the two-dimensional cAMP waves that mediate aggregation. Candidate tip inhibitors are Slug Turning Factor (STF), ammonia and adenosine (modified from Fig. 1 of ref. 20).

NH_3 , adenosine, and Slug Turning Factor (STF). Depending on whether tip activation or tip inhibition dominates the response the slug turns either towards or away from the light source.

Despite extensive research and the identification of many of the signalling components, understanding of the slug phototactic pathway is limited. It is clear however that the slug tip is extremely sensitive to directional light stimuli in its environment and is able to respond by orienting the slug. Thus phototaxis assays provide a means of studying the interaction between environmental signals and morphogenetic processes in the slug. This chapter is intended to assist the reader to measure phototactic responses accurately.

2. Materials

2.1. General Media

1. *Sterile saline*. 10 mM NaCl, 10 mM KCl, 2.7 mM CaCl₂.
2. *SM agar*. 4.1 mM MgSO₄, 16.2 mM KH₂PO₄, 5.8 mM K₂HPO₄, 1.0% Oxoid agar, 1.0% Oxoid bacteriological peptone, 0.1% Oxoid yeast extract, 1.0% glucose. Autoclave as two separate 500 mL solutions, one containing the glucose and one containing the other ingredients, then mix them aseptically after autoclaving, before pouring the plates. Separate autoclaving prevents caramelization of the glucose.
3. *Charcoal agar*. 1.0% Oxoid agar, 0.5% (w/v) activated charcoal, adjust pH to 6.5 with HCl or NaOH.
4. *Coomassie Blue stain*. 0.6% (w/v) Coomassie brilliant blue R (Sigma-Aldrich Inc.) in ethanol/acetic acid/water (5:1:4, v:v:v), used to stain slugs and slug trails (7).

2.2. Equipment

1. Spatula-style wooden toothpicks (*see* Fig. 2).
2. Matte black polyvinylchloride (PVC) boxes (98 mm external diameter, 22.6 mm external height, 2 mm thick PVC) were manufactured locally with a 4 mm diameter hole drilled through the side (*see* Fig. 2). Running 2 mm inside its perimeter, the base of each box has a 3 mm wide × 3 mm high lip which holds the base of a Petri dish in position when it is placed inside the box (*see* Fig. 2). The matte black colour of the box is designed to absorb stray light, which can interfere with phototaxis.
3. Clear PVC discs (84.5 mm diameter × 0.2 mm thickness) were manufactured locally as a large, single-batch special order.

3. Methods

3.1. Qualitative Tests

Qualitative tests are initially used to establish the general phototactic nature of a particular strain of *D. discoideum*. The number of amoebae used in these experiments is not calculated, yet a small amount must be used in order to avoid the effects of high cell density, which is known to impair phototaxis (7). For a more detailed analysis and collection of statistical data, a quantitative test should be performed.

1. Grow *D. discoideum* cells on 30 mL SM agar plates (*see* Note 1) containing a fresh *Klebsiella aerogenes* lawn as a food source at 21°C ± 1.0°C. The *D. discoideum* colonies arise as plaques on

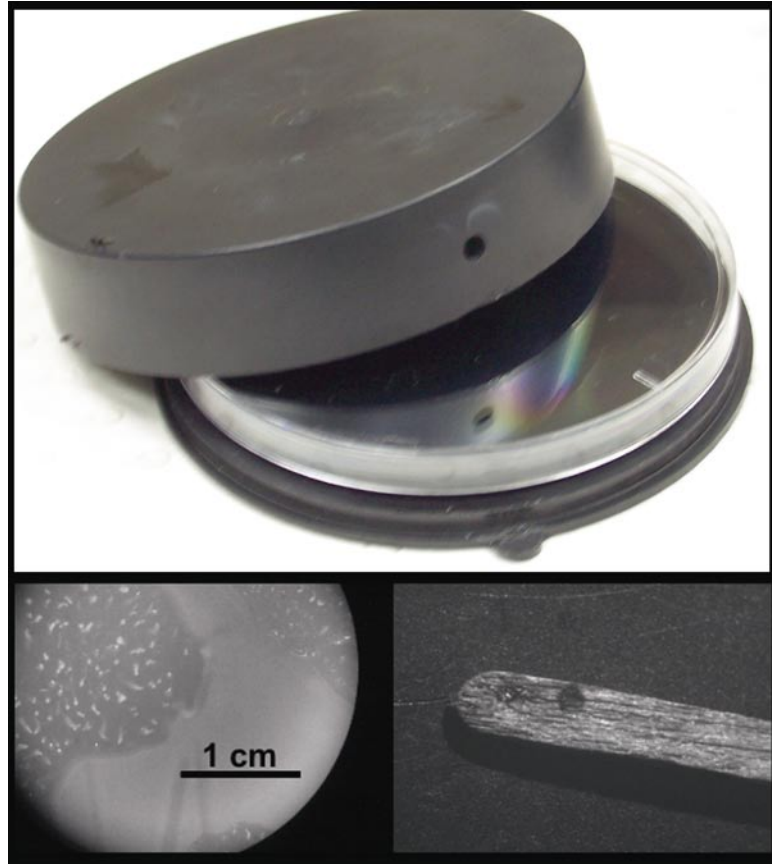


Fig. 2. Experimental setup for phototaxis. *Top panel:* An inoculated charcoal agar plate in a black polyvinylchloride box ready to be closed up for phototaxis. The plate is incubated in a lighted constant temperature room and light entering the hole in the side acts as the light source. *Bottom left panel:* Close-up of the edge of a growing *Dictyostelium* colony on a *Klebsiella aerogenes* lawn. About 5 mm of growth was scraped from the growing edge of the colony for inoculation onto water or charcoal agar. This illustrates the amount of amoebae to be inoculated – a larger inoculum may produce misleading results due to cell density effects. *Bottom right panel:* The blunt end of a spatula-style toothpick for scraping amoebae for inoculation. From Fig. 2 of ref. 20.

the *K. aerogenes* lawn. The amoebae are present at the edges of the colonies, and aggregation and differentiation occurs in the centre of the colonies, as the food source (*K. aerogenes*) has been depleted in these areas.

2. Scrape a small quantity of amoebae growing from the edges of *D. discoideum* colonies using a sterile, flat-edged toothpick and inoculate it onto a charcoal agar plate. Charcoal agar is used for phototaxis experiments, as the charcoal absorbs any stray light, which may interfere with slug phototaxis (6). If testing the effects of pharmacological agents on phototaxis the

use of water agar is preferred as the agents may be adsorbed by the charcoal. (Water agar is made as per charcoal agar with the exemption of charcoal). It is important not to inoculate too many amoebae onto the plate, as phototaxis is impaired at high cell densities due to the accumulation of high concentrations of STF (7). An approximate guide to the amount of cell growth that should be plated can be observed in **Fig. 2**. An inoculum this size provides just enough amoebae to form several (<10) slugs. Allow the plates to dry thoroughly before incubation (*see* **Note 2**).

3. After inoculation, place the charcoal agar plates into individual, covered, black PVC boxes with a 4 mm diameter hole drilled in one side (*see* **Fig. 2**). The plates are incubated in the same orientation at $21^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ for 48h facing a lateral light source (ambient room light in a constant temperature room is appropriate) (*see* **Note 3**). The starving amoebae aggregate to form slugs, which migrate over the agar surface, leaving behind a trail of collapsed slime sheath.

3.1.1. Quantitative Tests

Quantitative phototaxis tests use known densities of *D. discoideum* amoebae inoculated onto charcoal agar plates. This allows proper account to be taken of cell-density-dependent effects on slug behaviour and ensures that differences caused by mutational defects or other treatments, such as the addition of pharmacological agents, are not due simply to different cell densities.

1. Grow *D. discoideum* cells as in qualitative tests and make a suspension in sterile saline of approx 2×10^6 *D. discoideum* amoebae/mL using cells scraped from this growth plate. In a separate tube prepare a milky suspension of bacteria (appearance similar to fat-reduced milk) harvested with a sterile loop from a fresh *Klebsiella aerogenes* lawn (less than 1 week old).
2. Inoculate two plates with 0.1 mL of each of the bacterial and the amoebal suspensions (*see* **Note 4**). The inoculum is spread evenly over the entire surface of the plate and allowed to dry in the laminar flow hood, and finally the plates are incubated for 48 h at 21°C or until the lawns are clearing (*see* **Note 5**).
3. Harvest the amoebae from the clearing mass plates using 5 mL sterile saline per plate and a sterile glass spreader to gently scrape the amoebae from the plate surface, and suspend them in the saline. Wash the cells free of bacteria by repeated centrifugation at the lowest speed in a bench-top centrifuge ($\sim 600 \times g$ for 3 min). After the final wash, retain the amoebal pellet as the high-density, neat suspension. Prepare a 1:1,000 dilution (typically 10 μL in 10 mL) in sterile saline for cell counting later. The neat amoebal suspension should contain approx 1×10^9 amoebae per mL.

4. Prepare aliquots of a series of standard dilutions (usually 100%, 80%, 60%, 40%, 20% and 10%) in sterile saline and use them immediately for inoculation onto each 30 ml charcoal agar plate (20 μ L onto 1 cm²) (*see* [Note 6](#)). Duplicate plates for the 100%, 80%, 60% and 40% dilutions are prepared. The lower dilutions (20% and 10%) contain fewer amoebae and therefore produce fewer slugs, so 4–6 plates should be made from each of these dilutions. This ensures that enough slugs are produced to facilitate statistically accurate measurements of the slug behaviour. It is important to inoculate the amoebae in the centre of the plate, as gas diffusion into and out of the Petri dish leads to the development of radial gradients of ammonia and other gases that may influence slug behaviour ([1](#)). Ammonia itself is a slug repellent that accumulates in the air space between slug tips and physical barriers such as the Petri dish wall, with the result that slugs will tend to steer away from the edges of the plate. The 1-cm² area is measured (*see* [Note 7](#)) and the amoebae are spread evenly over this area using a pipette tip held at a low angle to the surface to spread the inoculum evenly.
5. Allow the agar plates to dry thoroughly in a laminar flow hood, as amoebae in a wet inoculum will not undergo multicellular development.
6. Place the charcoal agar plates into individual covered black PVC boxes with a drilled 4 mm diameter hole on one side and incubate them in the same orientation at 21°C $\pm$ 1.0°C for 48 h facing a lateral light source (fluorescent room light). The starving amoebae aggregate to form slugs, which migrate over the agar surface, leaving behind a trail of collapsed slime sheath.
7. After the phototaxis plates have been inoculated, determine the cell counts that were used by using a haemocytometer to count cells in the separate counting dilution that was made earlier. This means that exact densities are not controlled beforehand but are measured after the plates have been set up. However, the standard nature of the procedure with the use of the packed cell pellet as the dense suspension ensures that the densities are in approximately the required range.
8. To achieve a permanent record of the trails, use clear PVC discs to blot the plates – slugs, fruiting bodies, and slime trails adhere to the plastic discs. Stain the discs for 5 min with Coomassie Blue and then rinse them gently in running water (*see* [Note 8](#)). Use the stained discs to digitize the data, which are analysed statistically as described in Fisher and Annesley ([20](#)). Examples of digitized qualitative wild-type and mutant trails are shown in [Fig. 3](#).

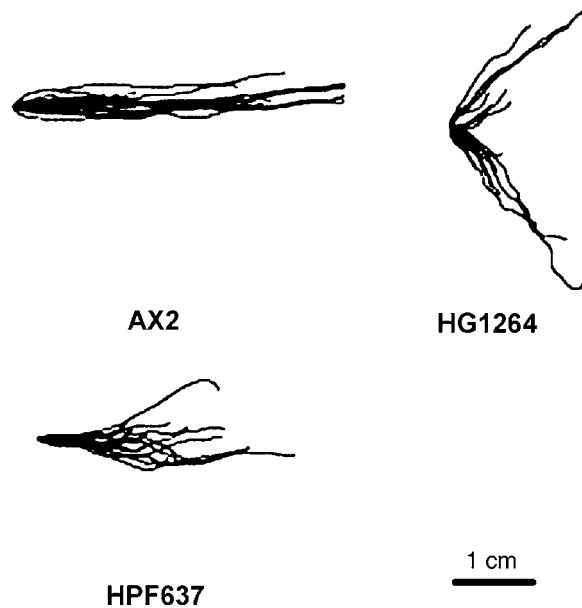


Fig. 3 Digitized qualitative slug trails. The slug trails of a wild-type strain (AX2) and two phototaxis mutants – HG1264 (filamin null mutant) with a severe phototaxis defect and HPF637 (HG1264 expressing a filamin form that lacks segment 1 of the rod domain) exhibiting a more subtle phototaxis defect (21). The light source is to the right of the figure.

4. Notes

1. The plates are thick so that they support growth of a luxuriant bacterial lawn and therefore a correspondingly high density of amoebae in the growing edge of the *Dictyostelium* colony.
2. It is worthwhile to check the plates after several hours for the formation of a water droplet at the inoculum site. If this happens, the plate should be allowed to dry in the laminar flow cabinet and the incubation then continued. Droplet formation sometimes happens, especially on freshly prepared plates, and if not corrected would prevent aggregation.
3. It is worthwhile, when establishing phototaxis assays in a new location, to carry out some control experiments using containers for plates that do not provide any entry for light. If the slugs migrate in an oriented manner on these plates, then there are temperature or other gradients present that can control the behaviour and influence the outcome of phototaxis experiments. In such cases, it is useful to try several locations in the room or incubator until one is found that lacks significant asymmetric stimuli.
4. To achieve the most even spread possible, the amoebae are inoculated after the bacteria.

5. The optimum stage for harvesting cells is when clearing is well advanced in the central regions of the plates, but there are still no signs of aggregation. However, plates can still be used if some aggregates have begun to form in limited areas of the plate.
6. The use of volumes of agar lower than this produces thinner plates, with the result that higher concentrations of metabolites accumulate in the agar, and phototaxis begins to be impaired at lower cell densities.
7. This is done by visual comparison with a template placed alongside the plate being inoculated.
8. By placing the discs face-down onto the surface of the Coomassie Blue staining solution, rather than immersing them, the intensity of the background staining of the plastic is halved.

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Chapter 5

Electrotaxis and Wound Healing: Experimental Methods to Study Electric Fields as a Directional Signal for Cell Migration

Guangping Tai, Brian Reid, Lin Cao, and Min Zhao

Summary

Electric fields were measured at human skin wounds over one and half centuries ago. Modern techniques have verified and greatly extended our understanding of the existence of endogenous wound electric fields. In virtually all wounds studied, disruption of an epithelial layer instantaneously generates endogenous electric fields. As electric fields have the intrinsic property of being vectorial, it has long been proposed that these fields may serve as a directional signal guiding cell migration in wound healing. We have established several experimental systems to study the guidance effects and mechanisms of electric fields on cell migration. Most types of cells migrate directionally in a small electric field, a phenomenon called galvanotaxis/electrotaxis. Remarkably, electric fields of strength equal to those detected at in vivo wounds direct cell migration and override some other well-accepted coexistent guidance cues such as contact inhibition. The naturally occurring endogenous electric fields therefore may be an important signaling mechanism that regulates directional cell movement in vivo. Applied electric fields may have a potential clinical role in guiding cell migration in wound healing. The magnitude and direction of the electric field can be more precisely and quickly changed than most other guidance cues such as chemical cues. Application of electric fields thus offers a robust experimental system for study of directional cell migration with extensive flexibility. We present a brief review of the background and describe the experimental system for studying electrotaxis.

Key words: Electrical fields, Endogenous electric signals, Directional cell migration, Wound healing

1. Introduction

Endogenous electric fields were measured at human skin wounds in the mid-nineteenth century. German physiologist Emil Du-Bois Reymond, using a self-built galvanometer, measured electric currents of $\sim 1 \mu\text{A}$ flowing out of a skin wound on his own finger (1, 2).

Following the development of modern techniques, such as the glass microelectrode and vibrating probe, endogenous wound electric fields have been confirmed at virtually all wounds that have been measured. For example, the stump of regenerating newt limb drives a large current that flows out of the stump. Electrical currents of 10–100 $\mu\text{A}/\text{cm}^2$ create a steady voltage drop of ~ 60 mV/mm within the first 125 μm of the extracellular space, and this endogenous electrical activity is essential for regeneration (3). When guinea pig skin is wounded, a large steady electric field (EF) arises immediately and persists for hours at the wound edge (4). These fields measure ~ 140 mV/mm and have been proposed to play an important role in directing cell migration in wound healing (5). Development of the vibrating probe technique has allowed noninvasive measurements with great temporal and spatial resolution of endogenous electric currents at rat corneal wounds and ocular lens (6–9). More recently, microneedle arrays have been developed and used to measure transdermal skin potentials in human subjects (10), and a bioelectric imager is able to “visualize” electric potential changes at skin wounds (11).

The electric fields at wounds are generated because of the trans-epithelial potential (TEP). Mammalian (including human) and amphibian skin maintains an electric potential difference across the epithelial layers with the basal side positive relative to the apical side. Wounding creates a low resistance path through the otherwise highly electrically resistant epithelium, causing a “short circuit” at the wound site. The TEP thus drives electric current flow out of the site of short circuit that occurs at the skin lesion (Fig. 1). The electric currents (flow of positive charge) are orientated toward the wound, being highest at the wound edge where active directional cell migration occurs (7) (Fig. 2). Therefore, the wound electric fields are well positioned to guide cell migration (5, 8, 12–14).

Many types of cells migrate directionally in an electric field, a phenomenon called electrotaxis or galvanotaxis (15, 16). It should be noted that not all types of cells migrate in the same direction. Cells derived from the same tissue can migrate in opposite directions. For example, corneal epithelial cells and osteoblasts migrate toward the negative pole (cathode), while corneal stromal fibroblasts and osteoclasts migrate to the positive (anode) (17–21). At the same field strength, other cells such as human skin fibroblasts may take much longer to respond (19, 20). Importantly, for corneal epithelial cells, the electric signals are an overriding guidance cue. In an electric field of the strength that has been detected at in vivo wounds, corneal epithelial cells follow the direction of the field and ignore other cues, such as initial injury stimulation, contact inhibition release, population pressure and other coexisting cues (21).

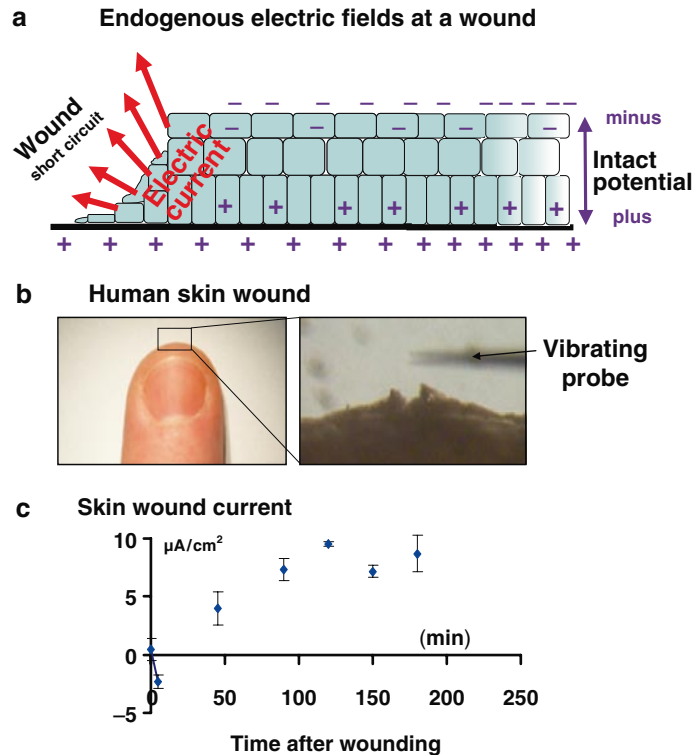


Fig. 1. Endogenous electric fields at epithelial wounds. (a) Schematic showing electric current flow (arrows) driven out the wound site by the transepithelial potential difference. (b) Small cut on human finger tip (left) and vibrating probe measuring current at the wound site (right). (c) Electrical currents at human finger wound. (b, c) are modified from (6).

Electrotactic migration is not (at least not exclusively) mediated by chemotaxis. When chemical gradients in an electric field are disrupted with a continuous flow of culture medium perpendicular to the electric field vector, cells respond just as well to the electric field (20). G-protein coupled receptor signaling is essential for chemotaxis of many types of cells (22). *Dictyostelium discoideum* cells with a G β subunit null mutation lose their chemotactic response while maintaining motility (23). Nevertheless, G β null mutants still respond to applied electric fields by directional migration, albeit less robustly than wild-type cells (20, 24). Therefore, electrotaxis and chemotaxis can be decoupled.

We describe here techniques for electrotaxis experiments, with specific methods for corneal epithelial cells and differentiated HL60 cells (20). We also include the principles we use for analysis of electrotaxis from timelapse images.

Directional cell migration at a wound

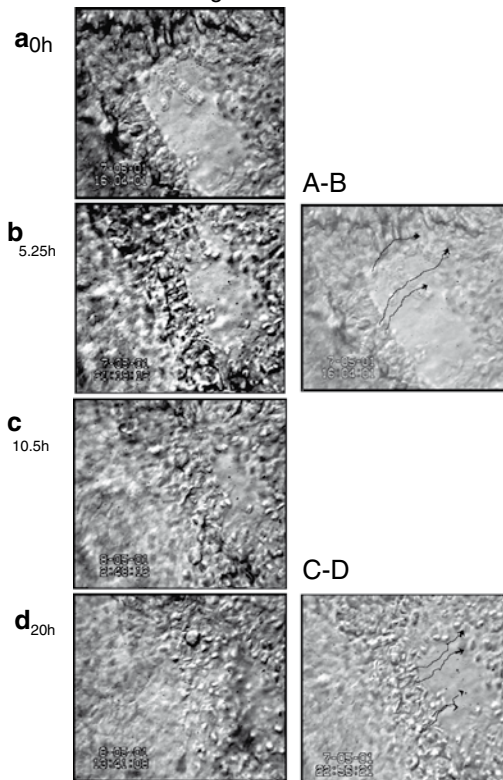


Fig. 2. Directional cell migration at a wound of stratified corneal epithelium in cornea organ culture. Timelapse images of the wound during healing (times at left). (a, b) Migration trajectories of three cells in the healing wound (between images a and b) as tracked by MetaMorph software. (c, d) Migration trajectories of three cells in the healing wound (between images c and d). (See online video: <http://www.fasebj.org/cgi/content/full/17/3/397/F4/DC1>, Modified from (15)).

2. Materials

2.1. Cell Culture

1. *Plastic tissue culture vessels*. Dishes, 35 × 10 mm and 100 × 20 mm; flasks, various sizes (Falcon, Wiesbaden, Germany).
2. *Corneal epithelial cell culture medium*. Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM/ml L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml kanamycin, 7.5 µg/ml fungizone, 10% fetal bovine serum (FBS) (Gibco, Paisley, Scotland, UK).
3. HL60 cells (a human promyelocytic leukemia cell line).
4. *PHAkt-GFP HL60 cells*. HL60 cells stably expressing PHAkt-GFP (pleckstrin homology domain of Akt tagged with the green fluorescent protein).

5. *HL60 cell culture medium*. RPMI 1640 supplemented with 25 mM HEPES, 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin G, 50 µg/ml gentamycin.
6. Dimethyl sulfoxide (DMSO) for HL60 cell differentiation (25).

2.2. Electrotaxis Chamber and Components of Electric Circuitry

1. Electrotaxis chambers are prepared in tissue culture dishes. Strips of glass coverslip (0.7 cm × 2.2 cm) are cut from a No.1 cover glass (6.4 cm × 2.2 cm; or 2.2 cm × 2.2 cm) (Chance Proper, Warley, England) with a diamond knife. The strips of cover-glass are sterilized in an autoclave. Sterilized silicone grease (Don Corning, MS4) is used to glue the glass coverslip strips onto the base of the cell culture dish (Fig. 3a) (see Note 1).

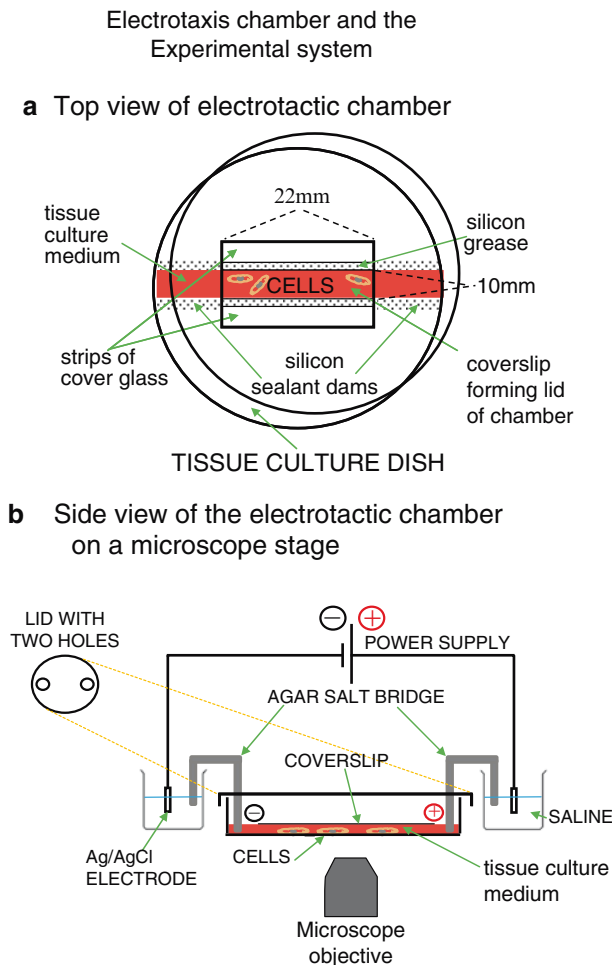


Fig. 3. Electrotaxis chamber and electric circuitry. (a) Chamber constructed in a plastic tissue culture dish, viewed from above. (b) Side view showing DC power supply, Ag/AgCl electrodes and agar-salt bridges that carry the voltage from the beakers to the two small medium reservoirs at the end of electrotaxis chamber. (Modified from (26)).

2. *Steinberg's solution*. 58 mM NaCl, 0.67 mM KCl, 0.44 mM $\text{Ca}(\text{NO}_3)_2$, 1.3 mM MgSO_4 , 4.6 mM Trizma base, pH 7.8–8.0.
3. Agar bridges for electrotaxis. The agar-salt bridge is a glass tube approximately 13 cm long with inner diameter of ~3mm (Fisher, UK) which is bent into a U-shape by melting in a bunsen flame. Agar (1–2%), dissolved by boiling in Steinberg's solution, is used to fill the glass bridge to form an electrically conductive tube. The agar-filled glass bridges are used to connect the electrical power supply, via silver/silver chloride electrodes and Steinberg's solution in beakers, to the cells in the electrotaxis chamber (**Fig. 3b**) (*see Note 2*).
4. *Modified Hank's Buffered Salt Solution (mHBSS)*. 150 mM NaCl, 4 mM KCl, 1.2 mM MgCl_2 , 10 mg/ml glucose, and 20 mM HEPES, pH 7.2.
5. Power supply capable of providing stable, adjustable DC output (up to 100 V).
6. Voltage (or volt/ohm) meter for measuring voltage strength in the electrotaxis chamber.

2.3. Western Blot

1. For collecting protein samples for Western blot, we use a similar electrotaxis chamber but of larger dimension: 64 mm × 25 mm × 0.2 mm. Caution should be taken to ensure that the chamber is of even depth in order to maintain a constant voltage gradient across the large chamber.
2. *Lysis buffer*. 10 mM Tris-HCl of pH 7.5, 50 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 1% Triton X-100, 30 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Boehringer Mannheim).
3. *SDS-PAGE*. 6% SDS-PAGE ready gel (Invitrogen), nitrocellulose membranes (Invitrogen), Coomassie Blue as a loading control (Bio-Rad), Ponceau S for detection of transfer efficiency, blocking reagent is 5% milk in PBS, or TBS (pH 7.4) with 0.1% (w/v) Tween 20 (Sigma).
4. Antibodies for Western blot analysis: antipan and antiactive Akt, antipan and antiactive extracellular signal-regulated kinases 1/2 (ERK1/2) (Cell signaling, USA); appropriate species of horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Amersham, UK).

2.4. Timelapse Video Microscopy

1. Microscope: inverted microscope equipped with CCD camera.
2. *Timelapse imaging system*. A computer connected to the camera to record timelapse images. We use image capture and analysis software MetaMorph.
3. Heated 37°C environment chamber with CO_2 on the microscope. This is optional depending on cell types. For *Dictyostelium* cells, room temperature will suffice (**20**).

We have used four different methods for video microscopy to study electrotaxis. Any image capture system which can take images at regular timepoints (timelapse images) is appropriate. An automated system is better but in most cases is not essential. For other options of timelapse image recording *see* **Notes 3–5**.

3. Methods

3.1. Preparation of Cells in the Electrotaxis Chamber

3.1.1. Preparation of Corneal Epithelial Cells in the Electrotaxis Chamber

1. Obtain fresh bovine eyes from a local abattoir and refrigerate (4°C) until use (usually within 2–10 h) (**27, 28**).
2. Remove blocks (~3 mm²) of limbus corneal epithelial layer (with part of stroma) near the conjunctiva under sterile conditions and transfer into a dish containing Dispase II (1.2 IU/ml) in calcium- and magnesium-free PBS (with antibiotics penicillin 250 IU/ml, streptomycin 250 mg/ml, gentamycin 0.5 mg/ml, Fungizone 7.5 mg/ml), and incubated at 37°C for 1.5 h.
3. Peel off epithelial layer in sterile saline under dissection microscope.
4. Trypsinize the epithelial layer in sterile saline containing 0.25% trypsin for 5–7 min at 37°C with intermittent gentle shaking.
5. Stop trypsinization by adding 10–15 ml DMEM (supplemented as described in **Subheading 2.1**) to deactivate the protease.
6. Centrifuge twice at ~160 × *g* for 5 min and resuspend cells in DMEM to a cell density of 14–24 × 10⁶ cells/ml. Cells are ready for seeding in the electrotaxis chamber.
7. Place approximately 1 ml of cell suspension in the electrotaxis chamber. We use fewer than 1 × 10⁵ cells per 6.4 cm × 1 cm chamber for single cell migration studies. 1–2 × 10⁷ cells/chamber of the same size is used for monolayer wound healing experiments.
8. Place the chamber in a 37°C, 5% CO₂ incubator to allow time for the cells to settle and adhere to the base of the chamber. The incubation time depends on the type of cells. Corneal epithelial cells migrate actively ~4 h to 2 days after seeding (**Fig. 4**). Neutrophils take much less time (15–30 min) to adhere to the base and migrate.
9. Wash gently with fresh medium to remove nonadhering cells.
10. Place a coverglass “roof” over the electrotaxis chamber and seal with silicone grease (or petroleum jelly, e.g. Vaseline) on each side, leaving the ends open (**Fig. 3a**) (*see* **Note 6**).

Electric field directs cell migration

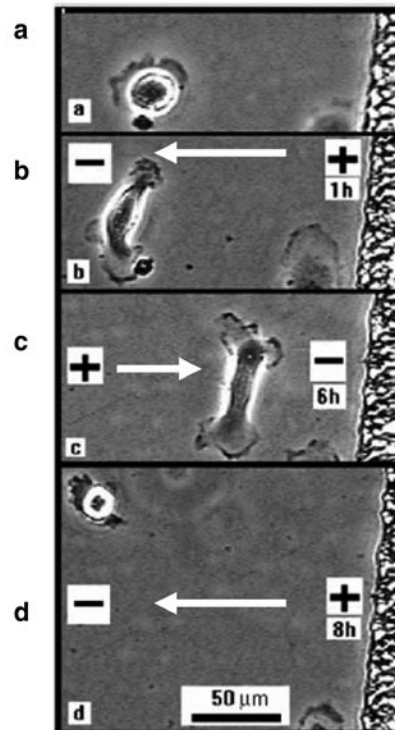


Fig. 4. Electric-field-directed migration of corneal epithelial cells – reversing polarity of the field reverses the migration direction. The reference scratch is to the right and EF polarity is shown by *white arrow* and +/– signs. (a). Before EF exposure; (b) 1 h in EF with the cathode to the left, then the polarity was reversed; (c) 6 h in EF with reversed polarity and the cell moved to the scratch (now the cathode side), then the polarity was reversed again; (d) 8 h in re-reversed EF, the cell moved away from the scratch toward the new cathode side, although by that stage its shape had changed. Cultured in DMEM with 10% FBS, at 150 mV/mm. (Modified from (26)).

3.1.2. Preparation of HL60 Cells and Phakt-GFP HL60 Cells in the Electrotaxis Chamber

1. Culture HL60 or PHAkt-GFP HL60 cells in suspension in flasks for 4 days using complete RPMI1640 medium until they reach a density of $\sim 1.5 \times 10^6$ cells/ml.
2. Centrifuge at $\sim 160 \times g$ and resuspend in 18 ml fresh antibiotic-free complete RPMI1640 medium (lacking G-418, gentamycin, and Fungizone).
3. Add 2 ml 13% DMSO solution and incubate at 37°C with 5% CO₂ for 6–7 days without changing the medium. Successful differentiation can be confirmed by checking for the polarized, elongated cell morphology of neutrophil-like cells.
4. Wash differentiated cells once in 10 ml RPMI 1640/25 mM HEPES medium.
5. Resuspend at a concentration of 3×10^6 cells/ml in modified HBSS (mHBSS).

6. Seed cells onto a sterile no. 1.5 acid-washed glass coverslip with an electrotaxis chamber similar to **Fig. 3**.
7. Incubate the cells on the coverslip at 37°C, 5% CO₂ for 20 min.
8. Remove nonadherent cells by two washes with mHBSS.
9. Add a roof of number 1 cover glass on top of the chamber and seal with silicone grease. Final dimensions of the chamber, through which the electric current passes, are 22 × 10 × 0.2 mm for both cell migration experiments and fluorescence imaging of living cells.

3.2. Application of Electric Fields

3.2.1. Preparation of Agar-Salt Bridges

1. Autoclave glass bridges before use (can be kept in sterile condition for weeks wrapped in aluminum foil).
2. Dissolve 1% agar in Steinberg's solution by heating to ~95°C.
3. Cool down the melted agar for 5 min at room temperature before filling the bridges.
4. Fill the glass bridge with a disposable plastic Pasteur pipette with one continuous flow of agar to prevent air bubbles. The agar will solidify in ~1–2 min depending on the ambient temperature.

3.2.2. Connection of the Electrical Circuitry (Fig. 3b)

Depending on the purpose of the experiment, the electrotaxis chamber is placed on the microscope stage (for imaging cell migration) or simply on the lab bench for protein sample collection. If the chamber is placed on the microscope, align the chamber so that the cathode and the anode are on the left and the right so that the electric field lines run horizontally as viewed down the microscope and recorded in the imaging system.

1. Set the power supply to the off position.
2. Connect the power supply positive and negative outputs to the silver/silver chloride wires in the beakers with crocodile clips.
3. Place the agar bridges to connect the beakers to each end of the electrotaxis chamber. Two holes in the cell culture dish lid made by cutting through with a hot scalpel blade will minimize evaporation of culture medium during long experiments (**Fig. 3b**).

Freshly prepared agar bridges are suggested for each experiment. A check of the electrical connections is made before proceeding to the next step (*see Note 7*).

3.2.3. Application of a Direct Current Electric Field

1. Place two measuring electrodes in the medium reservoirs at the ends of the electrotaxis chamber.
2. Set the voltage dial on the power supply to minimum and turn on the power supply.

3. Slowly turn up the voltage until the required voltage is read on the meter. For example, if the experiment requires a field strength of 100 mV/mm, then the reading on the meter should be 2.2 V in a chamber of 2.2 cm (22 mm) long: $2.2/22 \text{ mm} = 0.1 \text{ V}$ (100 mV). The actual output from the power supply will be much higher than this due to the voltage drop caused by the resistance in the beakers and agar bridges.
4. Adjust the strength of the electric field to any desired level during the experiments. Measure the voltage across the chamber regularly to avoid fluctuation (*see* [Note 8](#)).
5. If required, add fresh medium, drugs, or chemical agents via the reservoir at the end of the chamber. We remove old medium and add fresh medium by the “push-and-pull” technique, in which a pipette is placed in one reservoir to remove the old medium while at the same time another pipette places fresh medium in the other reservoir at the other end of the chamber. With practice this is a reliable and effective technique (*see* [Note 8](#) and [9](#)).

3.3. Imaging Cell Migration in an Electric Field

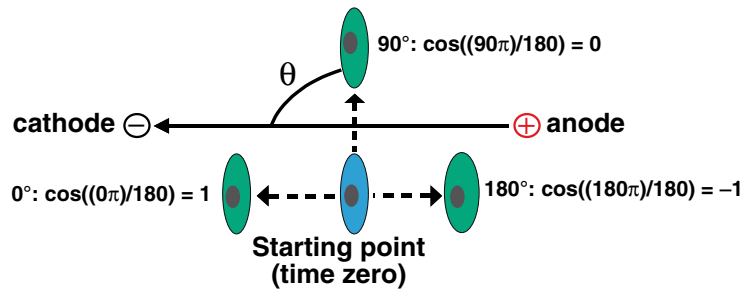
1. Adjust the orientation of the electrotaxis chamber so that it is aligned with the microscope stage with the anode and cathode at left and right, respectively, and so that the electric field flows ‘horizontally’ as viewed down the microscope. This is an important step as the angle measurement will not be accurate if the chamber is not properly aligned. Make a note of the positions (left or right) of anode and cathode.
2. View the cells in the electrotaxis chamber under the microscope to find optimal field(s) for timelapse imaging. Some imaging systems with a computer-controlled motorized microscope stage have the facility to program the positions of several fields of view into the imaging software, and the software will then automatically take an image at each position at each timepoint. If a focus motor is present then each field can also have its focus point programmed. Alternatively, some imaging software has autofocus which checks the focus of each field at each timepoint before taking an image. Stability of focus is important in these long timelapse experiments; the environment chamber should be brought up to temperature well in advance of starting the experiment, and the microscope should be placed on a solid, stable bench, or a heavy vibration-isolation table if possible.
3. Select the time interval you want between acquisition of images, and the total length of the experiment (or total number of images) in the programmable image acquisition software. Start timelapse image recording, checking that the software is taking the correct fields and they are in focus. If taking images manually, use a timer to measure time intervals. Timelapse recordings are made at 5-min intervals for corneal epithelia cells and 5-s intervals for differentiated HL60 cells.

3.4. Quantitative Analysis of Cell Migration

We use the following methods to analyze and quantify directional cell migration, the migration rate, and cell directedness (26, 29). Directedness and migration rate are quantified by tracing the positions of cell nuclei. For each field of view photographed during the experiment, a “movie” or “image stack” is made consisting of all the images taken at that field of view. This movie shows the movement of the cells in that field of view during experiment. Individual images can be taken out for a montage to show cell migration. For cell-tracking analysis, each cell, at the starting point (time zero) of electric field application, is assumed to be at the origin of a theoretical graph, with the X axis parallel to the field direction (cell at starting point in Fig. 5a).

Quantitative analysis of cell migration

a **Directedness = $\cos((\theta\pi)/180)$**
Mean Directedness = $\Sigma n(\cos((\theta\pi)/180))/n$



b **Scatter plots of directional cell migration**
Cells in field move toward cathode

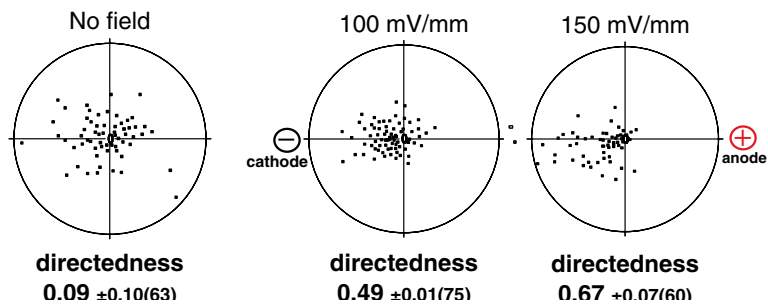


Fig. 5. Analysis and quantification of cell migration. (a) A schematic diagram shows the method for calculation of migration directedness of a cell. The cell begins at the starting point at time zero. If the cell moves directly toward the cathode (in this case to the left), the angle θ between the line of directionality and the field line is 0; therefore, $\cos((0\pi)/180) = 1$. If the cell moves perpendicular to the field line, the angle θ is 90, and $\cos((90\pi)/180) = 0$. If the cell moves toward the anode on the right, the angle θ is 180, $\cos((180\pi)/180) = -1$. The average directedness value of a group of cells (n = cell number) gives a quantification of how directionally the population of cells migrated in an electric field: Mean directedness = $\Sigma n(\cos((\theta\pi)/180))/n$. (b) Scatter plots show positions of cells after 5 h in an electric field, with the start points of all the cells placed at the origin in the center.

Imaging software such as Leica Image Analyzer, MetaMorph, or ImageJ allows the user to carry out the analyses described below. ImageJ is freely available from the NIH website (<http://rsbweb.nih.gov/ij/>).

3.4.1. Semiautomatic Analysis

1. Open image taken before EF application (starting point; time zero) and then each subsequent image at the same field of view until the end of the experiment (a 4-h experiment with 5-min time interval will have 49 images). If the software has each image in a separate window then merge them all into one window to make a “movie” or “image stack,” e.g., in ImageJ select the Image menu, go to submenu Stacks and select Images to Stack.
2. Some image analysis software programs have a “position selector” which allows the user to click on an object of interest and save the position as co-ordinates. Choose a cell and run the movie through to ensure the cell does not migrate out of the image field. Mark the position of the cell (the center of the nucleus is a good point to use) at each time point. Thus we have a list of co-ordinates which describe the cell’s movement during the experiment. A straight line can be drawn from the start position of the cell to the end position (**Fig. 5a**). The angle (θ) formed between the line of cell migration and the X-axis is used for the following quantification.

Migration directedness (based on cosine of angle θ) shows how directionally a cell migrated within the field, where θ is the angle between the EF vector (usually the horizontal) and a straight line connecting the start and end position of a cell (**26**). A cell moving directly along the field lines toward the cathode would have a θ angle of 0 and so a directedness of 1 ($\cos((0\pi)/180) = 1$); a cell moving directly toward the anode would have a θ angle of 180 and so a directedness of -1 ($\cos((180\pi)/180) = -1$). A cell moving at right angles to the field would have a θ angle of 90 and so a directedness of 0 ($\cos((90\pi)/180) = 0$) (**Fig. 5a**). A mean directedness value of a cell population close to 0 represents random cell movement. A directedness value higher than 0 and approaching 1 means that more cells migrated toward the cathode, while a value less than 0 and approaching -1 shows that more cells migrated toward the anode (**Fig. 5b**). Therefore, the average directedness of a population of cells gives an objective quantification of how directionally cells have moved.

Migration rate is calculated by the displacement distance divided by time (length of experiment). The displacement distance is the length of a straight line drawn from the position of a cell at the starting point (time zero) to its location at the end of the experiment. The total displacement distance is divided by the length (time) of the experiment to give the migration rate. For

example, displacement distance of 200 μm during a 6-h experiment gives migration speed of $200/6 = 33.3 \mu\text{m/h}$.

The scatter plot represents the cell distribution at the end of the experiment (*see* Fig. 5b). The origin of the graph is the position of every cell at the start (time zero), and each data point on the graph is where individual cells migrated to during the experiment. This gives a direct and visual representation of cell migration; directional cell migration and the effect of voltage on the directionality can be readily appreciated from the scatter plots.

3.4.2. Automatic Analysis and Quantification of Electrotaxis in Metamorph

1. Open individual timelapse pictures or stacks (movies) of the timelapse images.
2. Choose the object-tracking function. An object-tracking module is used to track individual cell movement automatically.
3. Follow the instruction of the software to track cells. The software will automatically record the positions (co-ordinates) of the cells. The software yields a number of parameters of cell migration (described later) (24). ImageJ freely available software from NIH website is also able to track cell movement (*see* <http://rsbweb.nih.gov/ij/>).

Trajectory analysis visualizes the track of cell migration (arrow lines in Fig. 2 a–d). We set the tracking mode to “semi-interactive,” so when the computer loses the track of a cell, it asks for user input to identify the object it is trying to track. Quantitative raw data are generated automatically and can be saved as a .csv file which can be opened with Microsoft Excel. We have formulated templates in Excel to convert the tracking parameters into directedness and migration rate as illustrated in Fig. 5.

Migration rate is analyzed with the following 3 parameters. Trajectory speed (T_t/T) is the total length of the migration trajectory of a cell (T_t) divided by the given period of time (T). Displacement speed (T_d/T) is the straight-line distance between the start and the end positions of a cell (T_d) divided by the time (T). Displacement speed along the X -axis (D_x/T) is a cell's displacement distance along X -axis (D_x) divided by the time (T).

3.5. Western Blot Analysis: Assay of Protein Phosphorylation in an Electric Field

1. Culture cells in larger electrotaxis chamber to maximize the protein yield. The procedure is the same as in Subheading 3.1.1 except that the chamber is larger.
2. Starve cultures in serum-free culture medium for 8 h (20).
3. Complete the electric circuit and expose to an EF of defined strength for a planned duration. The steps are the same as in Subheadings 3.2.2 and 3.2.3.
4. Turn off the electric field at defined time points. Rinse cells with cold PBS and collect cell lysate after treatment with lysis buffer. Samples can be stored at -20°C .

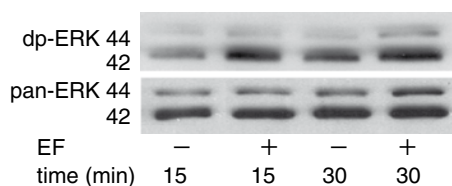
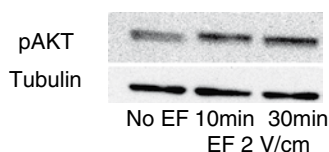
a Electric fields induced phosphorylation of ERK**b** Electric fields activate AKT

Fig. 6. **(a)** Electric field-induced dual phosphorylation of ERK1/2. Cells were starved overnight in serum-free medium. Fresh medium with or without 10% serum was introduced in the electrotaxis chamber and cells exposed to 150 mV/mm for the periods of time shown. Three independent experiments were done with similar results. (Modified from (21)). **(b)** Akt in keratinocytes and neutrophils is activated in an electric field.

- Resolve the protein lysate by using 4–12% SDS-PAGE, then transfer onto nitrocellulose membrane.
- Probe with antiactive ERK 1/2 antibody (1:5,000), and donkey antirabbit secondary antibody with horseradish peroxidase (HRP; 1:10,000) detected by ECL (enhanced chemiluminescence).

We used this approach to show that exposure of corneal epithelial cells to electric fields induced rapid and sustained phosphorylation of extracellular-signal-regulated kinase 1/2 (ERK) (Fig. 6a). We also find that other kinases such as p38 mitogen-activated kinase, Src, and Akt (Fig. 6b) are activated in keratinocytes and neutrophils after exposure to an electric field (20).

3.6. Confocal Microscopy of Protein Phosphorylation in an Electric Field

- Wash cells with PBS after exposure to an electric field for a defined time.
- Fix cells in 4% paraformaldehyde and permeabilize with 0.05% Triton X-20.
- Dilute antibodies in PBS with 1% BSA and use as follows: monoclonal anti-EGFR (epidermal growth factor receptor) at 1:80; anti-dp-ERK 1/2 (dual-phosphorylated ERK 1/2) 1:50; sheep antimouse IgG FITC conjugate 1:128 (F2266) and 1:250 (F2883), respectively.
- Incubate cells with primary antibodies at 37°C for 30 min.

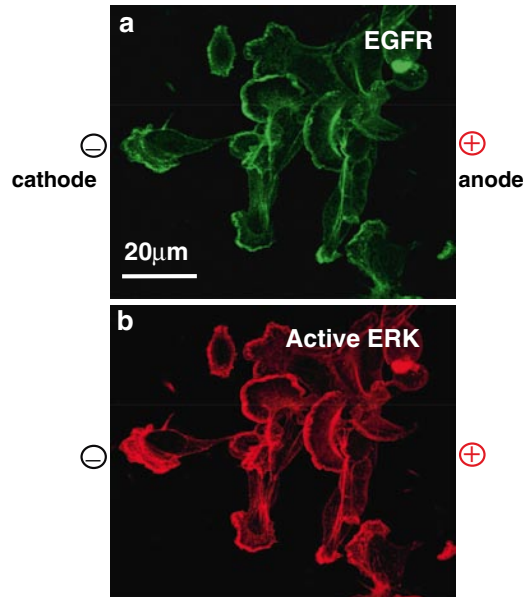


Fig. 7. An electric field induces cathodal distribution of EGF receptor and activation of ERK. Immunofluorescence micrographs show the asymmetry of (a) EGFR and (b) dual-phosphorylated ERK1/2. Cells were exposed to an electric field of 150 mV/mm for 1.5 h. (Modified from (21)).

5. Wash the cells twice with PBS, then incubate with secondary antibody and rhodamine-phalloidin for 30 min. For negative control staining, use IgG or the secondary antibodies only.
 6. Mount the slides in Vectashield and view with a confocal microscope (e.g., Bio-Rad MRC 1024).
- Using this method, we showed that exposure of cells to electric field induces asymmetric distribution of EGFR and dp-ERK 1/2 (Fig. 7).

3.7. Fluorescence Timelapse Video Imaging of Cell Migration in an Electric Field

1. Culture, differentiate, and seed PHAkt-GFP HL60 cells in electrotaxis chamber as detailed in **Subheading 3.1.2**.
2. Filter mHBSS with 0.2-μm filter and replace the mHBSS prior to timelapse experiments to improve optical clarity.
3. Mount the electrotaxis chamber on a heated microscope stage set at 37°C.
4. Prepare agar bridges, complete the electric circuit, and apply electric fields to the cells as detailed in **Subheading 3.2**.
5. Start timelapse imaging. We use a scientific-grade cooled charged-coupled device (CCD camera) on a multiwavelength MetaMorph

system. Cells are imaged using a 40 or 60×1.4 N.A. Zeiss fluoar lens and $n = 1.518$ immersion oil using GFP channel.

6. Switch the polarity of the electric fields to record the reversal of directional cell migration.

Using this protocol, we found that electric fields activate PI3 kinase at the cathodal side of the cells and upon reversal of the field polarity, a new site of activation of PI3 kinase formed, and new leading edge and cell migration toward the new cathodal side followed (**Figs. 8 and 9**).

Electric fields direct migration of a HL60 cell

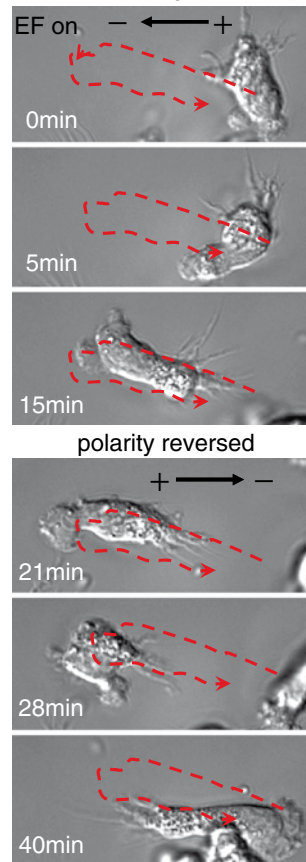


Fig. 8. An electric field directs migration of a differentiated HL60 cell. Differentiated HL60 cells are cultured on fibronectin-coated glass slides and exposed to an electric field. The field direction is reversed after 15 min. The cell migration is directed by the field. The *dotted line* illustrates the trajectory of cell migration.

An electric field induces polarized activation of PI3 kinases

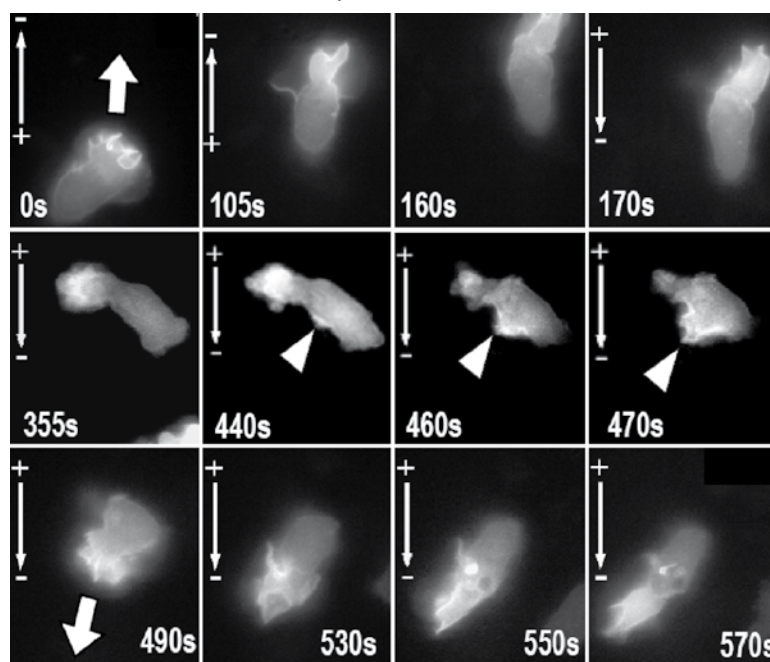


Fig. 9. Electric-field-directed activation of PI3 kinases. Differentiated PHAkt-GFP HL60 cells are plated in an electrotaxis chamber on a coverslip, and PI3 kinase activation visualized using PHAkt-GFP as a probe. EF induced directed cell migration toward the cathode and rapid PI3K activation in the leading edge of HL60 cells (0–105 s). When polarity of the EF is reversed, PHAkt-GFP redistributed toward the new leading edge and new membrane protrusion and cell migration ensue (440–470s; arrowheads). (See online movie: <http://www.nature.com/nature/journal/v442/n7101/extref/nature0-04925-s6.mova> from (20) supplementary figures).

4. Notes

1. It is important to maintain high resistance through the chamber for electric currents to minimize the Joule effect. We make the chamber very shallow, $\sim 200 \mu\text{m}$ in depth. The width and the length of the chamber can be adjusted as required, but the depth has to be minimized. The electric field is applied along the long axis of the chamber. The field strength is calculated by V/L , where V is the total voltage across the chamber and L the length of the chamber. The field strength is normally expressed as V/cm or mV/mm .

Some commercially available slides with built-in chambers can be adapted for electric field application, for example, the μ -slide from ibidi GmbH Integrated BioDiagnostics, Germany (<http://www.ibidi.de/>). The thin chamber built on the slide can be used for culture of dissociated cells and application of electric fields through the built-in wells at the ends of the chamber.

2. Glass bridges and coverslips for electrotaxis chamber and the greases are sterilized by autoclave before use. Other materials and equipment that contact the cultures need to be sterile.
3. Timelapse video recorder. We used a commercially available timelapse video recorder (VT-1200E Time Lapse VCR) connected to a camera on the microscope camera port. The interval of image taking can be defined and the timelapse images recorded on an SVHS video tape. An extra step is needed - to convert the video from the tape to digital video. The analog output from the VCR can be easily digitized with a computer into digital videos for further analysis.
4. Portable computer-controlled camera-based automatic timelapse imaging system. This is a cost-effective system. With many imaging software packages available, it is neither difficult nor expensive to set up timelapse or real-time imaging of cell migration. We use an inverted microscope (e.g., Nikon CK2-TRP with phototube), equipped with a USB-compatible digital camera (such as Moticam 1000 Microscope CMOS Camera). Moticam is a high-speed color microscope camera (CMOS) with Maximum Resolution of $1,280 \times 1,024$ Pixels, image output: USB 2.0 and 480 Mbps (<http://www.sapltd.co.uk>). After installation of the USB driver for the camera, the image capture software offers up to 96 h of continuous timelapse image capture. The advantage of this portable USB microscope camera is that it is fully sealed and water proof, and can be set up in a standard CO₂ cell culture incubator for long-term experiments (1–6 days). These long experiments are best done in the incubator where the stable conditions best suit the cells. Most commercially available chamber systems offer good temperature and CO₂ control. This is a simple, low price, automated image capture system; it can be set up in any lab at low cost but with full automatic image capture capacity.
5. A basic timelapse image capture system is a simple manual system controlled by a PC. This can be coupled to any inverted microscope. The PC can be installed with software such as Leica Image Analyser (Leica, Q500MC, Cambridge) or other image capture software for image capture. Cell migration can be recorded through taking images at defined time points manually. The disadvantage of such a system is it is time consuming and work intensive. Each picture needs to be taken manually at every time point.

If multiple fields need to be imaged, we make thin grids with fine epidermal needles on the base of the chamber to facilitate identification of individual fields. Images of cells can be taken using the grid of scratch marking as a reference. Markers on the scratch line can be used to identify each field, trace cells, and measure the angle of cell migration at each time point (26). **Figure 4** shows the same field taken at different timepoints with the reference scratch to the right as a marker to relocate the field.

6. When adding the roof on the chamber, care must be taken to avoid air bubbles being trapped in the chamber, which can alter the current density locally and causes uneven electrical resistance in the chamber. Reservoirs of culture medium are present at each end of the chamber to connect the agar-salt bridges – the two ends of the chamber are enclosed by silicone sealant dams, high enough (3–4 mm) to contain enough medium (1–2 ml) and to avoid overflow (**Fig. 3a**). The final dimensions of the chamber, through which current is passed, are 64 mm × 10 mm × 0.5 mm, or 2.2 mm × 10 mm × 0.5 mm (**Fig. 3**). These procedures should be done in a laminar flow hood to maintain sterility.
7. Good electrical connections are essential for the electrotaxis experiments. Air bubbles are easily trapped in the electrotaxis chamber or in the agar bridge. It is necessary to check carefully the connections before switching on the power. Short circuit due to overflow of the medium outside the chamber needs to be carefully avoided.
8. Maintain a stable voltage. When starting experiments, start from minimum voltage and turn the power supply up to the field strength required for the experiment. This avoids exposing the cells to excessive voltage. Electrodes with thin wires can be left in the two reservoirs at the ends of the chamber for easy and continuous monitoring of the voltage. Regular checks in the chamber with the volt meter should be made to monitor voltage across the chamber.
9. For experiments which need a long electric field application or drug addition, medium in the chamber can be changed using a push–pull technique with Pasteur pipettes. Care should be taken not to touch the dish or the chamber to avoid moving the imaging field.

Acknowledgments

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Chapter 6

Chemotropism During Yeast Mating

Peter J. Follette and Robert A. Arkowitz

Summary

Virtually all eukaryotic cells can grow in a polarized fashion in response to external signals. Cells can respond to gradients of chemoattractants or chemorepellents by directional growth, a process referred to as chemotropism. The budding yeast *Saccharomyces cerevisiae* undergoes chemotropic growth during mating, in which two haploid cells of opposite mating type grow toward one another. We have shown that mating pheromone gradients are essential for efficient mating in yeast and have examined the chemotropism defects of different yeast mutants. Two methods of assessing the ability of yeast strains to respond to pheromone gradients are presented here.

Key words: Chemotropism, Mating pheromone, Bud scar, Gradient, Zygote

1. Introduction

In the budding yeast *Saccharomyces cerevisiae*, haploid cells respond to mating pheromone peptides *via* G-protein-coupled receptors and their associated heterotrimeric G-proteins. Activation of these receptors results in cell cycle arrest, transcriptional activation *via* a MAP-kinase cascade, and polarized growth toward the mating partner (1). While mating pheromone itself is sufficient to induce the cell cycle arrest, transcriptional activation, and cell shape changes (to form a pear-shaped cell called a shmoo) that occur during mating, the mating pheromone gradient that is generated during the mating process is necessary for the oriented growth toward the mating partner (2–5); this chemotropic process is similar to that required for axonal guidance (6).

We have shown that Cdc24, the activator of the Rho G-protein Cdc42, is necessary for chemotropism during yeast mating (3). Two other proteins, the heterotrimeric G-protein (7–9) and the cyclin-dependent kinase inhibitor Far1 (2, 5), are similarly required

for chemotropic growth during mating and form a complex with Cdc24 (3, 10, 11). Recently, several studies have revealed that the MAP-kinases Fus1 and Kss1 are also important for yeast chemotropic growth (7, 12, 13). Chemotropic growth is necessary for efficient yeast mating, and mutants defective in chemotropism, such as *cdc24-m1* or *far1-H7* mutants, have an approximately 20–50-fold reduction in mating efficiency (5, 11).

Chemotropism in yeast can either be measured directly, e.g., in haploid cells in the absence of a mating partner (4, 13), or indirectly during the mating process (2, 5). For direct measurements of chemotropism, the orientation of the mating projection of haploid cells can be observed relative to an artificial pheromone gradient generated by a micropipet (4). Very recently, a microfluidic chamber has also been used to generate a linear pheromone concentration gradient resulting from passive diffusion between two parallel microchannels (12, 13).

To indirectly evaluate the contribution of chemotropism during yeast mating, two approaches can be taken. First, the pheromone gradient can be abolished by adding exogenous mating pheromone to the mating reaction; this approach is referred to as the “pheromone confusion assay” (2). Alternatively, the position of growth can be determined relative to a fixed cellular landmark (namely, the bud scar) (5). One shortcoming of the pheromone confusion assay is that because the pheromone gradient is eliminated by the addition of exogenous mating pheromone, the resulting saturating levels of pheromone in the mating reaction may affect other processes in addition to the pheromone gradient. The second method relies upon determining the position of growth relative to the bud scar (5). During normal, chemotropic mating, cells orient their growth toward their mating partner, irrespective of the position of the previous bud site. In contrast, in the absence of chemotropism, the site of growth during mating is adjacent to the previous bud, following the axial bud site selection pattern in haploid cells (3, 5). Accordingly, in this method the bud scar is first stained in one mating type prior to mating, and then, once the stained cells have mated with cells of the opposite mating type, the position of growth in zygotes (two haploid cells that have fused to form a dumbbell-shaped diploid) is determined relative to the stained bud scar. Because chemotropism mutants are still capable of mating, albeit at reduced efficiency, it is possible to observe zygotes in mating assays between chemotropism mutants and wild-type tester cells.

2. Materials

2.1. Cell Growth and Labeling

1. YEPD rich medium (*see* Note 1) can either be purchased ready-made (for example from Sigma-Aldrich, St. Louis, MO; Clontech, Mountain View, CA; or MP Biomedicals, Solon, OH) or made

from individual components. YEPD medium is comprised of 11 g yeast extract, 22 g bactopectone, 55 mg adenine sulfate, and 20 g glucose per liter (*see* [Notes 2](#) and [3](#)). YEPD plates contain, in addition, 22 g agar per liter (*see* [Note 4](#)).

2. Synthetic dropout medium can either be purchased ready-made (for example from Sigma-Aldrich, Clontech or MP Bio-medicals) or made from individual components (*see* [Note 5](#)). Synthetic dropout medium is comprised of 8 g yeast nitrogen base, 55 mg adenine sulfate, 55 mg tyrosine, 55 mg uracil (for Ura⁻ strains), and 20 g glucose per liter. Synthetic dropout plates contain, in addition, 22 g agar per liter. A 100× concentrated amino acid dropout stock contains: 2 g arginine, 1 g histidine, 6 g isoleucine, 6 g leucine, 4 g lysine 1 g methionine, 6 g phenylalanine, 5 g threonine, and 4 g tryptophan per liter. Amino acids necessary for selection are omitted. Synthetic complete (SC) medium has no amino acids omitted from the dropout.
3. Calcofluor white Fluorescent Brightener 28 (M2R)(Sigma-Aldrich or MP Biomedicals) is made up as a stock solution of 10 mg/mL in water, and diluted solutions of 1 mg/mL (in water) are prepared from this stock and used for daily experiments (*see* [Note 6](#)).
4. α -factor mating peptide (*see* [Note 7](#)) is dissolved at 4 mg/0.6 mL 0.1 M sodium acetate, pH 5.2, and aliquots are stored at -20°C.

2.2. Quantitative Yeast Matings

1. Nitrocellulose membrane filters: MF-Millipore Membrane Filters, type HA, pore size 0.45 μ m, diameter 25 mm (Millipore, Billerica, MA).
2. Vacuum filter manifolds that accommodate up to, *e.g.*, 10–12 membrane filters (Millipore or Sigma-Aldrich).
3. Glass beads, ~4 mm diameter (Fisher Scientific, Pittsburgh, PA). Autoclave in glass bottles prior to use.
4. 37% formaldehyde solution.

3. Methods

We describe here two methods that can be used to assess the contribution of the mating pheromone gradient during yeast mating (1) eliminating the mating pheromone gradient by the addition of saturating amounts of exogenous mating pheromone, and (2) determining the position of growth during mating relative to a fixed landmark, namely the bud scar. The first method is based upon the fact that if a mutant is chemotropism defective, then abolishing the mating pheromone gradient by the addition of

saturating amounts of exogenous mating pheromone will have little or no effect on mating efficiency. Accordingly, the mating efficiency can be compared between a candidate chemotropism-defective mutant strain and a wild-type strain in the presence and absence of saturating levels of mating pheromone in the same conditions. The second method takes advantage of the fact that wild-type yeast cells mating in a chemotropism-dependent fashion grow toward their mating partners irrespective of the position of their bud scars, while cells mating in the absence of chemotropism (such as in a chemotropism mutant or when the mating pheromone gradient has been eliminated) orient their growth in a default pattern adjacent to the previous bud scar. In this method, the bud scars of a yeast strain are marked with calcofluor white prior to mixing with a mating partner. After allowing sufficient time for mating to occur, the mating mixture is examined by fluorescence and differential interference contrast microscopy, and the position of growth during mating is determined relative to the bud scars.

3.1. Mating Pheromone Confusion Assay

1. Wild-type and mutant yeast *MAT α* strains and a wild-type *MAT α* tester strain are grown overnight in selective medium and back diluted into 5 mL of rich medium (YEPD) to an OD₆₀₀ of 0.05–0.1 in the morning. Cells are then grown to an OD₆₀₀ of 0.6–0.8 (at least two doublings).
2. Volumes corresponding to 0.5 OD₆₀₀ of *MAT α* and *MAT α* strains are transferred to individual sterile microcentrifuge tubes. The cells are centrifuged at maximum speed in an Eppendorf centrifuge at room temperature for 10–20 s and an amount of the supernatants removed such that a 0.5-mL volume remains for each cell type. The *MAT α* and *MAT α* cells are resuspended in their respective microcentrifuge tubes and then their entire volumes are combined in a 50 μ L Falcon tube (or glass tube) in the presence or absence of 5 l of α -factor (4 mM). These mating mixtures are incubated at 30°C for 5 h with gentle shaking (100 rpm in orbital shaker).
3. Nitrocellulose membrane filters are rinsed with 5 mL of YEPD in a vacuum filter manifold. Subsequently, the mating mixtures are transferred to the membranes by filtration and the membranes rinsed twice with 10 mL YEPD. The filters are then removed with sterile Millipore tweezers (dipped in 70% EtOH and flamed) and placed in 2-mL microcentrifuge tubes containing 2 mL of SC medium. The tubes are sonicated in a sonication bath for 10 s at 8% maximum amplitude (*see* [Note 8](#)).
4. The filters are removed from the tubes with sterile Millipore tweezers and the mixtures serially diluted to produce 1:10, 1:100, and 1:1,000 dilutions (*see* [Note 9](#)) in 1 mL of SC medium in 1.5-mL microcentrifuge tubes. The tubes are

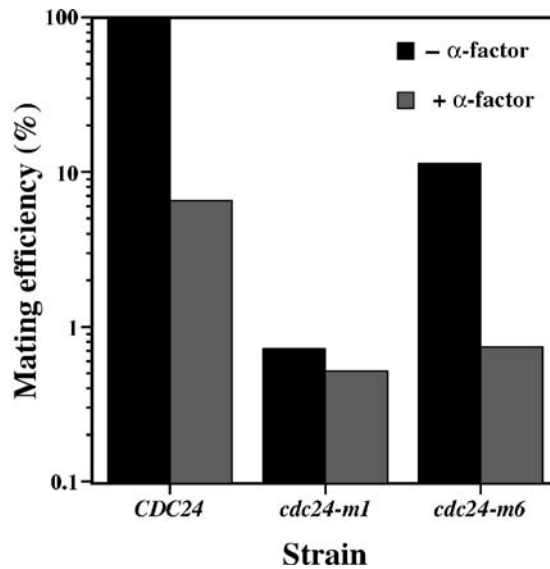


Fig. 1. Results of a Mating Pheromone Confusion Assay. The mating efficiencies of wild-type and mutant strains are determined in the presence and absence of α -factor. Both the wild-type (*CDC24*) and the chemotropism functional mutant (*cdc24-m6*) cells show strongly decreased mating in the presence of α -factor. The mating efficiency of cells carrying the chemotropism-defective allele *cdc24-m1*, however, is not affected by the pheromone (14). Copyright © American Society for Microbiology.

vortexed and 100 μ l aliquots plated on selective plates and YEPD plates (See Notes 10 and 11). For plating, the aliquots are spotted onto each plate and 10–15 sterilized glass beads added to the plate. After spotting and adding glass beads to several plates, the plates are stacked and slowly shaken back and forth, turning every $\sim$ 10 s. After shaking for 2–3 min and ensuring that the plates are visibly dry, the beads are poured into a waste container. The plates are then sealed with Parafilm, inverted, and placed in a 30°C incubator for 3–4 days.

- At the end of the incubation time, the colonies are counted and the mating efficiency calculated by dividing the number of colonies on the selective plates (diploids) by the number of colonies on the YEPD plates (haploids and diploids). If the cells are chemotropism functional, then the addition of saturating levels of α -factor should substantially reduce the mating efficiency, *e.g.*, by 10–20-fold. In contrast, chemotropism-defective cells should display a reduced mating efficiency relative to wild-type cells that is not significantly altered by the presence of exogenous pheromone (Fig. 1).

3.2. Position of Growth during Mating Assay

- Wild-type and mutant yeast *MAT α* strains and a wild-type *MAT α* tester strain are grown overnight in selective medium and back-diluted in the morning into 5 mL of rich medium

- (YEPD) to attain an OD_{600} of 0.1. The cells are then grown to an OD_{600} of 0.6–0.8 (at least two doublings).
2. 0.2–0.5 OD_{600} of *MAT α* cells are then incubated with 10 μ g/mL calcofluor white (*see* [Note 12](#)) in YEPD in a 15-mL Falcon tube for 5 min at room temperature and then washed three times with 10 mL YEPD by centrifugation ($2,000 \times g$ in a table-top centrifuge for 3 min).
 3. Nitrocellulose membrane filters (Millipore) are rinsed with 5 mL of YEPD in a vacuum filter manifold. Equal amounts (volumes corresponding to 0.1 OD_{600} each) of *MAT α* cells and of calcofluor-white-stained *MAT α* cells are combined by transferring them to a single sterile microcentrifuge tube, and YEPD is added to reach a final volume of 0.5 mL. This mixture is gently vortexed and then filtered using the manifold. The filters are subsequently rinsed twice with 10 mL YEPD, removed from the manifold with sterile Millipore tweezers (dipped in 70% EtOH and flamed), and placed on YEPD plates with the cells facing up (*see* [Note 13](#)). The plates (filter facing up) are then incubated at 30°C for 3 h.
 4. The filters are removed from the YEPD plates with sterile Millipore tweezers and placed in 2-mL microcentrifuge tubes containing 2 mL SC medium. The tubes are sonicated in a sonication bath for 10 s at 8% maximum amplitude. Sterile Millipore tweezers are then used to remove the filters from each tube, and the mating mixtures are serially diluted (1:10, 1:100, 1:1000 dilutions in 1 mL of SC medium in 1.5-mL microcentrifuge tubes) (*see* [Note 9](#)).
 5. The tubes are vortexed and 100 μ L aliquots spotted onto selective and YEPD plates (*see* [Note 10](#)) by agitation with 10–15 sterilized glass beads. After allowing the plates to dry and inverting them to remove the glass beads, the plates are sealed with Parafilm, inverted, and placed in a 30°C incubator for 3–4 days. Following this incubation time, the colonies are counted and the mating efficiency calculated by dividing the number of colonies on the selective plates by the number of colonies on the YEPD plates (*see* [Note 14](#)).
 6. In parallel, 0.5 mL of the mating mixture (undiluted) is transferred to a 1.5-mL microcentrifuge tube and fixed with the addition of 50 μ L of a 37% fresh formaldehyde solution. After 30–60 min at room temperature, the microcentrifuge tube is centrifuged at maximum speed for 30 s, the supernatant is carefully removed, and 0.1 mL of sterile PBS is added (*see* [Note 15](#)).
 7. The cells are examined by DIC and fluorescence microscopy. Cells (1–2 μ L) are spotted onto a microscope slide and a coverslip is placed on the drop. The slides are viewed by DIC microscopy (to locate cells) and fluorescence microscopy with

UV excitation (excitation bandpass filter: 340–380 nm, 400 nm dichroic, and emission longpass filter: 425 nm) to visualize the bud scars (*see* [Note 16](#)).

8. Dumbbell-shaped prezygotes and zygotes (frequently observed with a third lobe/bud protruding from the center) (*see* [Note 17](#)) are identified using DIC microscopy ([Fig. 2](#)). As only the *MAT α* cells have been stained with calcofluor white, only one half of the dumbbell-shaped prezygotes and zygotes should exhibit strong UV-excited fluorescence ([Fig. 3](#)). By using the fine focus control, the bud scar is visualized with UV excitation on the labeled half of the prezygotes and zygotes (*see* [Note 18](#)). Only cells with a single bud scar are counted, and the position of the bud scar relative to the site of cell fusion

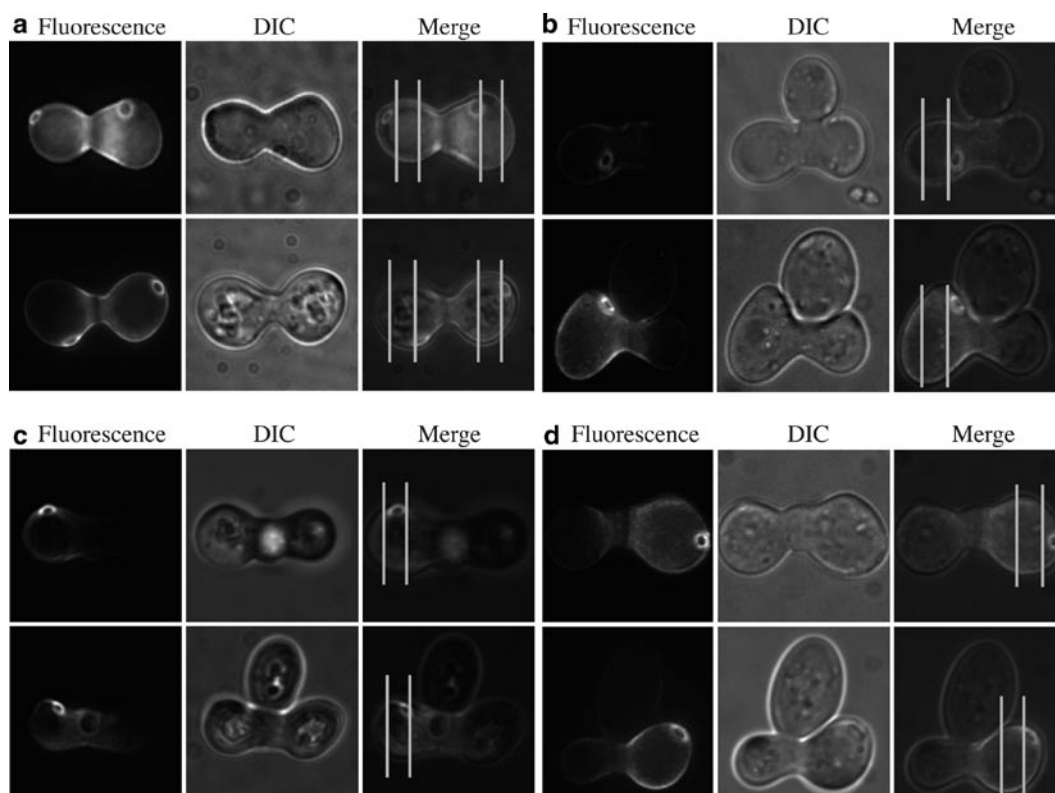


Fig. 2. Examples of calcofluor-white-stained bud scars in zygotes and prezygotes. **(a)** Mating mixtures were stained with calcofluor white, so both mating partners are labeled. Note bud scars which are distal and proximal to the position of growth during mating. **(b)** One mating partner was stained with calcofluor white and bud scars can be observed proximal to the site of growth. **(c)** One mating partner was stained with calcofluor white and bud scars can be observed medial to the site of growth. **(d)** One mating partner was stained with calcofluor white and bud scars can be observed distal to site of growth.

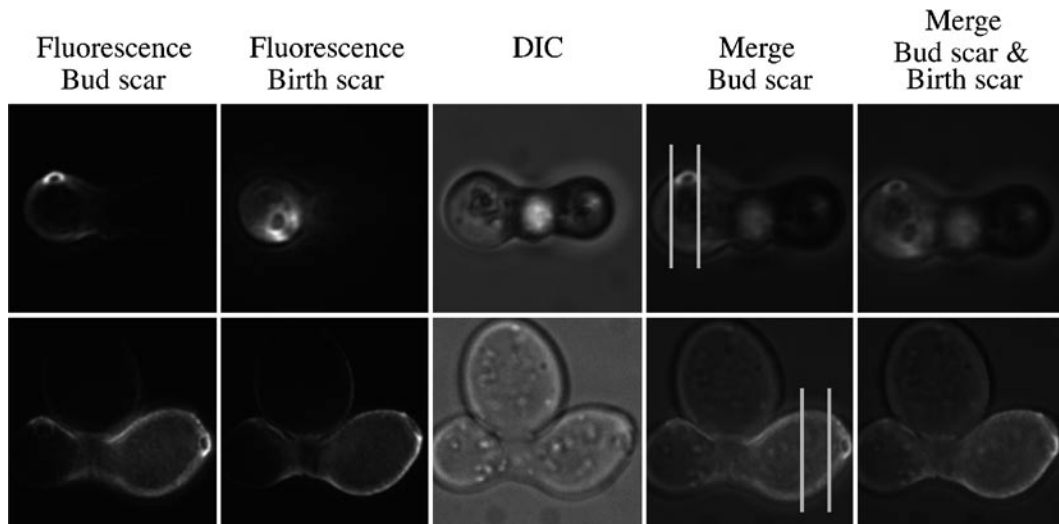


Fig. 3. Examples of zygotes with both bud scars and birth scars visible. Bud scars and birth scars are in different focal planes.

is scored: “proximal” for the one-third of the cell adjacent to the site of cell fusion, “medial” for the middle one-third of the cell, and “distal” for the one-third of the cell farthest from the site of cell fusion (*see* [Note 19](#)) (**Figs. 2 and 3**). Typically, 100–150 of such single-bud-scar prezygotes and zygotes are counted. It is also useful to confirm the position of growth in the *MAT α* cells that have not formed prezygotes or zygotes; however, this analysis is complicated by the fact that shmoos are not always clearly discernable, and if the strains do not form pointed shmoos it is frequently difficult to identify the tip of the projection (which is used to score the direction of growth relative to the bud scar) (*see* [Note 20](#)).

9. The percentages of cells with the bud scar proximal, medial, or distal to the site of fusion are then calculated for the wild-type and mutant strains. In general, in contrast to wild-type cells, chemotropism mutants mate by default and thus display growth sites during mating that are adjacent to the bud scar. As a result, while wild-type cells should have relatively equal percentages of proximal, medial, and distal bud scars relative to the site of fusion, chemotropism mutants should have a majority of cells with bud scars proximal to the site of fusion (**Fig. 4**). It should be noted that such analysis is not possible with mutants that perturb axial bud site selection.

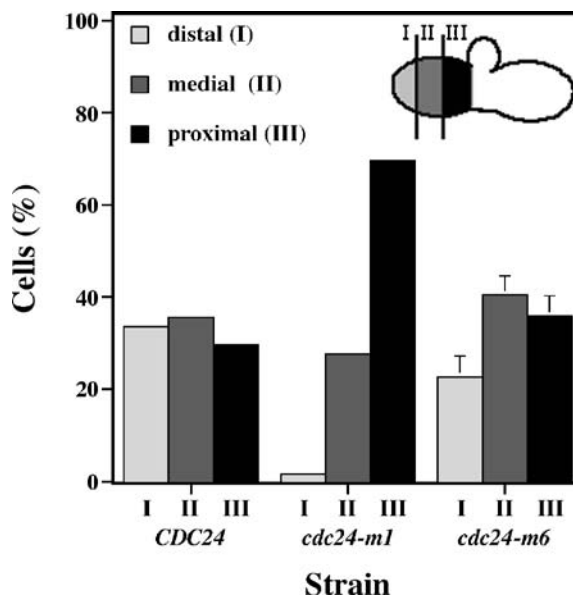


Fig. 4. Results of a Position of Growth During Mating Assay. Stained bud scars in wild-type and mutant zygotes and prezygotes were scored for their locations relative to the site of growth. Wild-type and chemotropism functional *cdc24-m6* mutant cells show essentially equivalent percentages of zygotes with bud scars that are distal, medial, or proximal to the growth site. In contrast, in the majority of chemotropism-defective *cdc24-m1* zygotes, the bud scars are proximal to the site of growth (14), Copyright © American Society for Microbiology.

4. Notes

1. All solutions should be prepared in water that has a resistivity of 18 MΩ-cm. This standard is referred to as “water” in this text.
2. YEPD medium is the same as YPD medium but also contains 50 mg/L adenine sulfate.
3. YEP (or YEP agar) is autoclaved and a one-tenth volume of a 20% (w/v) glucose solution (0.2 μm filter sterilized) is subsequently added.
4. Sterilized yeast medium is stored in 90-mL volumes without a carbon source (glucose) or amino acid supplements.
5. Yeast medium amino acid supplements can be stored for up to one year at 4°C in 10 mL aliquots in 15-mL Falcon tubes.
6. Calcofluor white solutions should be stored in a tube wrapped in aluminum foil (to protect from light) at 4°C. Stock solutions

should be made fresh each week as it has a tendency to precipitate if kept longer.

7. The 13-amino acid peptide α -factor can be purchased from commercial suppliers such as Sigma-Aldrich, Zymo Research (Orange, CA), or Pi Proteomics (Huntsville, AL), or can be purchased from peptide synthesis companies, which tends to be more economical.
8. The mating mixtures are sonicated to separate cells that may be adhering to one another.
9. The optimal dilutions for plating may vary depending on the particular strains used and on the presence or absence of pheromone. Generally, 100–200 colonies per plate are ideal for counting purposes.
10. The selective plates will lack multiple nutrients such that only diploid cells, *i.e.*, those carrying at least one marker from each haploid cell type, will be able to grow.
11. As fewer cells will be able to grow on the selective plates than on the nonselective YEPD plates, the optimal dilution will likely be lower for the selective plates than for the YEPD plates. Similarly, as the presence of α -factor reduces the mating efficiency of wild-type cells, the optimal dilution for wild-type cells on selective plates will likely differ depending on whether or not pheromone was present during the mating assay.
12. The calcofluor white concentration may be varied to obtain the best staining. Too high of a concentration can lead to substantial fluorescence throughout the zygote, complicating the analysis by making it difficult to definitively identify the originally labeled (*MATa*) cell.
13. Four filters can be placed on a single YEPD plate. It is important that there is good contact (*i.e.*, minimal air bubbles) between the filter and the agar on the plate.
14. If the mutant cells are chemotropism defective, then the mating efficiency should be substantially lower (*e.g.*, 20–50-fold) than in wild-type cells. This plating step is thus included to provide further confirmation that cells showing nonrandom growth relative to the bud scar are indeed chemotropism defective.
15. Fixed calcofluor-stained mating mixtures should be kept at 4°C, and we find that they can be counted up to one week after mating. With increased time, the unlabeled mating partner becomes fluorescent, and hence it is difficult to discern the two mating partners in prezygotes and zygotes.
16. We find that it is ideal to examine the cells using a 100× objective lens.

17. Zygotes are diploid cells formed by the fusion of two haploid cells. Prezygotes are intermediates of the mating reaction in which two mating partners have attached to one another, but the intervening cell wall is still present.
18. It is important to distinguish the birth scar, which is also visible upon calcofluor white staining, from the bud scar. The birth scar is typically larger ($\geq 1 \mu\text{m}$) and less strongly stained than the bud scar, which is usually visible as a clear ring of fluorescence (**Fig. 3**).
19. Note that all three regions for scoring (proximal, medial, and distal) fall within the portion of the zygote or prezygote that corresponds to the original cell, *i.e.*, the round or oval part of the dumbbell-shaped zygote (**Fig. 2**). Generally, no bud scars will be present within the thinner central (the “bar” of the dumbbell) part of the zygote or prezygote, which corresponds to the region of new growth during mating and typically exhibits less calcofluor white fluorescence compared to the rest of the cell.
20. To facilitate identification of the shmoo tip, a GFP fusion such as Spa2-GFP can be expressed in the strain. This allows positioning of the bud scar relative to the Spa2-GFP signal. As formaldehyde fixation substantially decreases the GFP signal, this analysis should be done in live mating mixtures kept on ice to prevent further mating.

Acknowledgments

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Chapter 7

Group Migration and Signal Relay in *Dictyostelium*

Paul W. Kriebel and Carole A. Parent

Summary

The ability of cells to migrate directionally in gradients of chemoattractant is a fundamental biological response that is essential for the survival of the social amoebae *Dictyostelium discoideum*. In *Dictyostelium*, cAMP is the most potent chemoattractant and the detection, synthesis, and degradation of cAMP is exquisitely regulated. Interestingly, as *Dictyostelium* cells migrate directionally, they do so in a head-to-tail fashion, forming characteristic streams. This group behavior is acquired through the relay of the cAMP signals to neighboring cells. This chapter describes experimental procedures used to obtain synchronized populations of chemotactically competent cells and to assess their streaming behavior. In addition, we provide a detailed account of the method used to measure the ability of chemoattractants to directly stimulate adenylyl cyclase activity. Together, these techniques provide a way to combine cell biological and biochemical approaches to the study of signal relay.

Key words: Adenylyl cyclase, G proteins, Chemotaxis, cAMP, Methods

1. Introduction

Cyclic AMP metabolism is essential for the survival of the social amoebae *Dictyostelium*. Upon starvation, these cells enter a developmental program where they migrate directionally and form an aggregate that eventually differentiates into a fruiting body composed of a head of resistant spores atop a stalk of vacuolated cells (1,2). The program is mediated by highly conserved signal transduction pathways, which are centered on the synthesis, secretion, and degradation of cAMP (3,4). A few hours after the initiation of starvation, *Dictyostelium* cells start to synthesize and secrete cAMP, which binds to G-protein-coupled receptors that specifically recognize cAMP (called cARs).

The binding of cAMP triggers a series of signal transduction events that regulate chemotaxis, control gene expression, and further activate the synthesis and secretion of cAMP. This last event, referred to as signal relay, allows cells to spontaneously aggregate. The enzyme responsible for the synthesis of cAMP is the adenylyl cyclase A (ACA). It shares homology with the mammalian G-protein-coupled adenylyl cyclase and is composed of two sets of six transmembrane domains followed by cytosolic loops where the catalytic sites reside. The binding of cAMP to its receptor leads to the dissociation of the heterotrimeric G protein into $G\alpha$ and $G\beta\gamma$ subunits. Interestingly, the activation of ACA does not depend on $G\alpha$. Rather it is mediated by $G\beta\gamma$ through the activation of PI3K, which in a Ras-dependent manner generates PIP_3 (4). This leads to the translocation of the PH domain-containing regulator CRAC (cytosolic regulator of adenylyl cyclase) from the cytoplasm to the plasma membrane, and, in concert with the TORC2 (target of rapamycin complex 2), the activation of ACA (5–7).

As *Dictyostelium* cells sense and migrate directionally to form aggregates, they align in a head-to-tail fashion and form chains of cells or streams. We found that this behavior requires the enrichment of ACA-containing intracellular vesicles at the back of polarized cells – a process that requires intact actin and microtubule networks and de novo protein synthesis (8,9). We proposed that this asymmetrical distribution of ACA provides a compartment from which cAMP is locally secreted to spatially attract neighboring cells. In this chapter, we outline various techniques used to visualize the ability of cells to stream during chemotaxis. We also provide a detailed account of the method used to directly measure the activation of ACA following chemoattractant stimulation as well as in response to G protein activation in cell lysates. Together, these assays provide the means to study the signal relay cascade in *Dictyostelium*.

2. Materials

2.1. Development of *Dictyostelium* Cells

1. *HL5 growth medium*. 20 g/l maltose (Sigma), 10 g/l proteose peptone (Cat#211684; BD Biosciences), 5 g/l yeast extract (Cat#212750; BD Biosciences), 3.6 mM Na_2HPO_4 , 3.6 mM KH_2PO_4 , and 41 μ M dihydrostreptomycin. Autoclave and store at room temperature.
2. *Developmental buffer, DB*. 5 mM Na_2HPO_4 , 5 mM NaH_2PO_4 of pH 6.2, 2 mM $MgSO_4$, 200 μ M $CaCl_2$. Store at room temperature.

3. *Phosphate buffer (PB)*. 5 mM Na_2HPO_4 , 5 mM NaH_2PO_4 , pH 6.2. Store at room temperature. Prepare as 10× stock and dilute as needed.
4. *DB Agar*. 1.5 g of Bacto agar (Cat#214010; BD Biosciences) in 100 ml of DB. Make fresh each day, microwave to dissolve agar, and store at room temperature.
5. *Wash buffer*. 20 mM Tris-HCl of pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20. Store at room temperature.
6. *Blocking buffer*. 20 g BSA in 500 ml wash buffer. Filter using 0.22- μm pore size filters and store at 4°C.
7. *Blotting buffer*. Combine blocking buffer (1 part) with wash buffer (3 parts) just prior to use.
8. *Protease inhibitors (PI)*. Add 1 tablet of Complete protease inhibitor cocktail (Roche) to 50 ml of PB. Store in 1-ml aliquots at -20°C.
9. *Sample buffer (5×)*. 25% (v/v) glycerol, 830 mM DTT, 260 mM SDS, 156 mM Tris-HCl of pH 7.5, 7.5 mM bromophenol blue. Store in aliquots at -20°C.
10. *Precast gels*. 18-well 4–20% Tris-HCl Criterion gel (Bio-Rad).
11. PVDF membranes (Bio-Rad).
12. Transfer buffer (Bio-Rad).
13. Secondary antibody: antirabbit IgG conjugated with horseradish peroxidase (Cat#NA934V; GE Healthcare).
14. ECL detection reagents (Amersham).

2.2. Group Migration Assays

1. HL5 (*see Subheading 2.1, item 1*).
2. Developmental buffer (*see Subheading 2.1, item 2*).
3. *DB Agarose*. 100 ml of DB and 0.5 g SeakemME agar (Cat#50010; Lonza). Microwave the suspension to melt agar into DB. Cool at room temperature until the suspension reaches 37°C. Make fresh prior to use.
4. 2 well-chambered coverglass (Lab-Tek II).
5. Femtotip micropipettes (Eppendorf, Germany).
6. Microinjector (Eppendorf, Germany).
7. Micromanipulator (Eppendorf, Germany).

2.3. Adenylyl Cyclase Activation Assay

1. HL5 (*see Subheading 2.1, item 1*).
2. Developmental buffer (*see Subheading 2.1, item 2*).
3. *PM*. 2 mM MgSO_4 in PB. Store at room temperature.
4. *Reaction mix*. 100 mM Tris-HCl, 1 mM ATP, 10 mM cAMP, 100 mM DTT, include 0.5 μCi [α - ^{32}P]ATP (MP

Biomedical) in each 20 μ l. Make just before use. Store at 22°C.

5. *Lysis buffer*. 20 mM Tris-HCl of pH 8, 2 mM MgSO_4 . Make just before use. Store at 4°C.
6. *GTP γ S stock solution*. 10 mM GTP γ S (Roche) in 10 $\times$ PB (*see Subheading 2.2, item 3*) and store at -20°C.
7. *Stop Solution*. 9 mM ATP, 10 mM cAMP, 2% SDS and 2 M Tris-HCl, pH 7.5 in dH_2O . Store in aliquots at -20°C.
8. Dowex AG 50W-X4 (200–400 mesh).
9. Poly Prep chromatography columns (Bio Rad).
10. Alumina
11. 1 M imidazole-HCl, pH 7.3.
12. 0.1 M imidazole-HCl, pH 7.3.
13. Nucleopore Track-Etch membranes, 5.0- μ m pore size (Whatman).

3. Methods

3.1. Development of *Dictyostelium* cells

3.1.1. Cell Development on Non-Nutrient Agar Dishes

1. Grow cells for the assay in HL5 to approximately 4×10^6 cells/ml at 22°C in Erlenmeyer flasks, shaking at 250 rpm (*see Note 1*).
2. Melt DB agar in a microwave 1 h before the assay (*see Note 2*).
3. Transfer 3 ml of DB agar to a 35 $\times$ 10 mm petri dish and allow it to solidify for 1 h at room temperature.
4. Transfer 1×10^7 cells from the shaking flask to a 50-ml conical tube, and pellet them by centrifugation at $500 \times g$ at room temperature for 5 min.
5. Wash the cells twice with 50 ml DB and finally resuspend them at 1×10^7 cells/ml in DB.
6. After the agar has solidified for 1 h, place 1×10^7 cells (1 ml) on the agar and swirl until the entire surface of the plate is covered (*see Note 3*).
7. Allow the cells to adhere for 5 min.
8. Tilt the plate and remove the liquid by gently aspirating (*see Note 4*).
9. Place the dishes on a flat surface and allow them to dry for 1 min (*see Note 5*).
10. Cover the dishes and store them in a closed box, layered with moist paper towels.

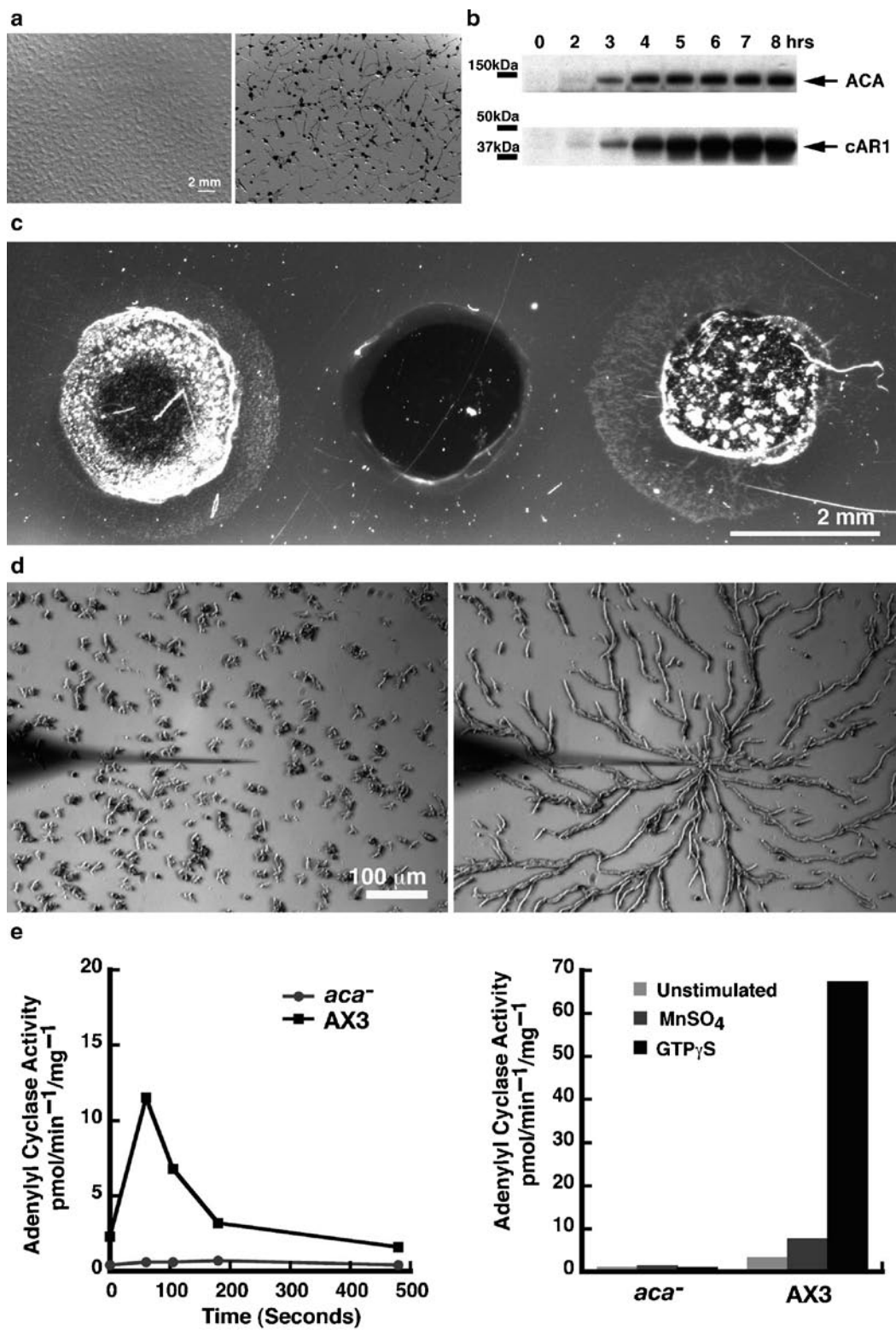
11. Incubate the dishes at 22°C overnight.
12. The entire developmental program can be recorded by taking pictures at various intervals. Best results are obtained at low magnification (2–10×) using a stereoscope equipped with a CCD camera controlled using an imaging software (we use the iVision-Mac software from BioVision Technologies) (*see* **Fig. 1**). Furthermore, the process can be recorded using time-lapse microscopy.

3.1.2. Cell Development in Non-Nutrient Buffer with cAMP Pulses

1. Grow cells as described in **step 1** of **Subheading 3.1.1**.
2. Transfer cells from the shaking flask to a 50-ml conical tube, and pellet them by centrifugation at $500 \times g$ at room temperature for 5 min.
3. Wash the cells once with 50 ml DB and finally resuspend them at 2×10^7 cells/ml in DB.
4. In order for the cells to reach the chemotaxis-competent stage in a synchronized fashion, transfer them to an Erlenmeyer flask and shake them at 100 rpm for 1 h. Then pulse the cell suspension with 75 nM cAMP every 6 min for 4–6 h (*see* **Notes 6 and 7**).

3.1.3. Assessing Development by Western Analysis

1. Prepare the cells for the assay as outlined in **steps 1–4** of **Subheading 3.1.2**.
2. Transfer 200 µl samples from the pulsing flask every hour after the initiation of starvation to 1.5-ml microcentrifuge tubes and centrifuge them at $7,500 \times g$ at room temperature for 2 min in a microcentrifuge. Aspirate the supernatant and resuspend the cells in 40 µl PB with PI and lyse them with 10 µl of 5× sample buffer. Vortex and quickly freeze the samples in dry ice and store at –20°C.
3. Thaw the samples and carefully load 35 µl into each well of a 4–20% Tris–HCl Criterion gel (*see* **Note 8**).
4. Run Criterion gels according to the manufacturer's procedure. Specifically, run them at 200 V until the dye front reaches the bottom of the gel (~ 1 h) and transfer them overnight at 4°C (at 30 V) to PVDF membranes using transfer buffer for Criterion blots containing 15% methanol.
5. Remove the membranes from the transfer apparatus, rinse them once with wash buffer, and add blocking buffer to cover the membrane. Place the membranes on a rocker at low speed for 1 h at room temperature or overnight at 4°C. Pour off the blocking buffer and rinse the membranes once in wash buffer.
6. cAR1 and ACA are good markers to assess the developmental state of aggregating cells. Probe the membrane with primary



antibody against cAR1 or ACA at a dilution of 1:2,000 in blotting buffer (*see* **Note 9**) on a rocker at low speed for 1 h at room temperature or overnight at 4°C (*see* **Note 10**).

7. Pour off the primary antibody into a tube and stored at 4°C, until used again. Add wash buffer to cover the membranes and place on a rocker at high speed for 10 min. Repeat the wash 3 times.
8. Cover the membrane with the secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase), diluted 1:2,000 in blotting buffer, and place it on a rocker at low speed for 1 h at room temperature.
9. Discard the secondary antibody and add wash buffer to cover the membranes. Place the membranes on a rocker at high speed for 10 min. Repeat the wash 3 times.
10. Drain the membranes onto a paper towel, add ECL detection reagents to cover the membrane, and incubate for 1 min, with gentle shaking at room temperature. Drain the chemiluminescent substrate from the membranes using a paper towel. Place the membranes face down on plastic wrap and fold the edges of the plastic wrap over the membrane. Place the membrane in a photographic film cassette and expose it to Kodak BR film until the band of interest is visible, but not overexposed (*see* **Fig. 1**).

3.2. Group Migration Assays

3.2.1. Self-Streaming Assay

1. Prepare cells for the assay as outlined in **steps 1–4** of **Sub-heading 3.1.2**.
2. Transfer 200 μ l of cell suspension from the pulsing flask to a 1.5-ml microcentrifuge tube. Centrifuge the samples at $7,500 \times g$ at room temperature for 2 min using a microcentrifuge. Aspirate the supernatant and resuspend the cell pellet with 200 μ l of PB. Before spotting the cells on a chamber slide, dilute the cells further in another microcentrifuge tube 1:10 with PB.
3. Plate the cells on a 2 well-chambered coverglass as 5 μ l spots to one side of each chamber.
4. Allow the cells to adhere for 5 min at room temperature and gently add 1.5 ml of PB.

Fig. 1. (a) Images of *aca*⁻ (left) and wild-type AX3 (right) cells 24 h after the initiation of development on non-nutrient agar plates. (b) Western blots depicting the expression of ACA (top) and cAR1 (bottom) during early development of AX3 cells. (c) Images showing AX3 (right) and *aca*⁻ (left) cells migrating under agar 1 h after plating. (d) Varel images of AX3 cells migrating to a micropipette filled with 1 μ M cAMP. (e) Adenylyl cyclase activity of AX3 and *aca*⁻ cells. Cyclic AMP-mediated activation is presented on the left and Mn²⁺- and GTP γ S-mediated activations are on the right. Kreibel and Parent, Fig. 1.

5. Transfer the loaded chambered coverglass to an inverted microscope and acquire DIC images with a 10× objective every 10 s using a CCD camera.
6. After ~20 min the wild-type cells will start to align in streams and form aggregates.

3.2.2. Streaming to a Micropipette

1. Prepare the cells for the assay as outlined in **steps 1–4 of Subheading 3.2.1.**
2. Transfer the loaded chambered coverglass to an inverted microscope and gently lower a Femtotip micropipette filled with 1 μ M cAMP to the bottom of the chamber using an Eppendorf micromanipulator. Generate the cAMP gradient from the micropipette using an Eppendorf microinjector. For this, set the pump at a P_i (injection pressure) of 198 and a P_c (compensation pressure) of 204. Alternatively the pump can be set to the continuous flow program with the pressure set at 80 (*see Note 11*).
3. Allow cells to migrate toward the micropipette for ~20 min while acquiring DIC images every 10 s using a 10× objective and a CCD camera (*see Fig. 1*).

3.2.3. Streaming Under Agar

1. Prepare cells for the assay as outlined in **steps 1–4 of Subheading 3.1.2.**
2. Centrifuge cells at $500 \times g$ and 22°C for 5 min and resuspend them at 1×10^7 cells/ml in PB.
3. To inhibit cAMP signaling and cell–cell communication, add 2 mM caffeine to cells.
4. Shake cells for 30 min at 22°C and 200 rpm.
5. Melt DB agarose in a microwave oven 1 h before the assay (*see Note 2*).
6. Allow the DB agarose suspension to cool to the touch and then add 1 mM caffeine.
7. Transfer 3 ml of DB agar to 35 $\times$ 10 mm polystyrene petri dishes and allow the agarose to solidify for 45 min.
8. Carefully core three identical wells out of the agar 1 mm apart using a Pasteur pipette attached to an aspirator.
9. Add 5 μ l of a 1 μ M cAMP solution in PB to the central well. A dose curve for cAMP can easily be performed (between 100 nM and 100 μ M cAMP).
10. Incubate the dishes at room temperature for 30 min to establish a cAMP gradient.
11. After aspirating the two outside wells to remove any debris, gently add 5 μ l of cell suspension and allow the cells to migrate.

12. Acquire static images after 15, 30, 60, and 120 min at low magnification (2–10×) using a stereoscope outfitted with a CCD camera (*see* **Fig. 1**). Alternatively, the migration of cells can be recorded using an inverted microscope.

3.3. Adenylyl Cyclase Activity Assay

This assay is based on the previously published procedure (**10**). It includes the use of ATP radiolabeled with ^{32}P , which has a half-life of 14 days. It is important to use the $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ within a week and a half after receiving it. Otherwise the radioactive levels become too low to perform the assay. Since ^{32}P is a β -emitter, adequate shielding has to be used to protect against exposure. Consult with your institution's radiation safety office to follow safety precautions for your work place regarding use, disposal, and storage of radioactive material.

3.3.1. Preparation of Dowex and Alumina Chromatography Columns

1. Wash Dowex AG 50W-X4 (200–400 mesh), a cation exchanger, with dH_2O in a graduated cylinder until the effluent is color free (*see* **Note 12**).
2. Make a 50% (v/v) Dowex to dH_2O slurry by pouring off the last dH_2O wash, estimating the volume of the remaining Dowex and adding an equal volume of dH_2O .
3. Transfer the slurry to a beaker and stir vigorously.
4. While stirring, transfer 2 ml of homogenous slurry to 0.8 cm × 4 cm Poly Prep chromatography columns.
5. Wash the columns once with 2 ml 1N HCl and store at room temperature.
6. Prepare alumina columns by the transferring 0.5–0.6 g of dry neutral alumina into a second set of Poly Prep columns.
7. Wash the alumina columns once with 12–15 ml of 1 M imidazole–HCl, pH 7.3, followed by 15–20 ml of 0.1 M imidazole–HCl, pH 7.3. After the columns are drained, store them at room temperature.

3.3.2. Cell Preparation

1. Prepare cells for the assay as outlined in **steps 1–4 of Sub-heading 3.2.3**.
2. Transfer cells to 50-ml conical tubes and centrifuge at $500 \times g$, 4°C , for 5 min.
3. Wash cells twice in 50 ml ice-cold PM and centrifuge at $500 \times g$, 4°C , for 5 min.
4. Resuspend cells at 8×10^7 cells/ml in ice-cold PM and store on ice (*see* **Note 13**).

3.3.3. cAMP-Mediated Adenylyl Cyclase Activation

This assay is used to measure adenylyl cyclase activity induced by the binding of cAMP to cAR1 in intact cells. Adenylyl cyclase

activity is typically measured 0, 60, 105, 180, 300, and 480 s after stimulus.

1. Prepare lysing syringes by inserting a folded Nucleopore membrane between a 1-ml syringe and the needle. One syringe/time point is required.
2. Transfer 2.5 ml of cells to a 6-ml plastic sample cup and shake at 200 rpm at room temperature.
3. Add 10 μ M cAMP to the cells shaking in the sample cup and take 300 μ l samples at 60, 105, 180, 300, and 480 s for assay as in **steps 3–6** (see **Note 14**).
4. Immediately after removing the 300- μ l sample of cells from the cup add the cells to a 1-ml lysing syringe loaded with 300 μ l of lysis buffer. Rapidly insert and press the plunger in the syringe lysing the cells by pushing the cells through the pores in the membranes. Collect the lysate in 12 $\times$ 75 mm glass tubes at 22°C.
5. Immediately transfer 200 μ l aliquots of lysate to two 12 $\times$ 75 mm glass tubes containing 20 μ l reaction mix and gently shake.
6. Allow the reaction to proceed for 1 min at 22°C and then stop the reaction by adding 100 μ l of stop solution to each tube.
7. Prepare assay blanks by adding 200 μ l of lysis buffer instead of cell lysates to reaction tubes.

3.3.4. Mn^{2+} and $GTP\gamma S$ -Mediated Adenylyl Cyclase Activation

This assay is used to measure adenylyl cyclase activity downstream of the receptor, by directly activating lysates with $GTP\gamma S$ – a non-hydrolyzable form of GTP – or $MnSO_4$ – an ATP chelator that provides a measurement of the intrinsic adenylyl cyclase catalytic activity. $MnSO_4$ measurements are therefore used as an alternative readout of ACA expression levels.

1. Prepare lysing syringes as outlined in **step 1** of **Subheading 3.3.3**.
2. Measure basal and Mn^{2+} stimulated adenylyl cyclase activity from the same cells prepared for the cAMP mediated assay (from **step 4** of **Subheading 3.3.2**). Transfer 600 μ l of cells on ice to a 1-ml lysing syringe loaded with 600 μ l of lysis buffer, and rapidly lyse the cells into a 12 $\times$ 75 mm glass tube on ice.
3. Incubate the lysates on ice for 4 min.
4. Immediately transfer 200 μ l of lysate each into four 12 $\times$ 75 mm glass tubes two of which contain 20 μ l of reaction mix and two, which contain 20 μ l of reaction mix and 40 mM $MnSO_4$ gently shake.

5. Allow the reaction to proceed for 2 min at 22°C and then stop the reaction by adding 100 µl of stop solution to each tube.
6. Measure GTPγS-mediated activation from cells prepared from **step 4 of Subheading 3.3.2**) by adding 300 µl of cells into a lysing syringe loaded with 300 µl of lysing buffer containing 80 µM GTPγS and 2 µM cAMP. The rest of the procedure is as outlined for the basal measurements.

3.3.5. Chromatography

1. Wash the Dowex columns twice with 10 ml of 1 mM imidazole, pH 7.0.
2. Wash the alumina columns twice with 10 ml of 100 mM imidazole, pH 7.5.
3. Prepare the samples for chromatography by adding 680 µl dH₂O to each tube.
4. Prepare radioactivity inputs by transferring 10 µl of a sample directly to a 7-ml scintillation tube. This is done twice on randomly chosen samples. Store inputs at room temperature until samples are ready for counting on the scintillation counter.
5. Decant samples into the Dowex columns, allowing them to soak in and drain from the columns.
6. Wash the Dowex columns twice with 1 ml of 1 mM imidazole, pH 7.0. In this step, [³²P]cAMP is bound to the columns and >99% of the [α-³²P]ATP is eluted.
7. To load the alumina columns, place the Dowex columns on top of the alumina columns, add 5 ml of 1 mM imidazole, pH 7.0, to the Dowex columns and allow them to drip through the Alumina columns. In this step [³²P]cAMP is eluted from the Dowex columns and absorbed quantitatively on the Alumina columns.
8. Finally, elute the [³²P]cAMP by adding 3 ml of 100 mM imidazole, pH 7.5, to the alumina columns and allow them to drip directly into 7-ml scintillation counting vials. Cap the scintillation vials and count using the ³²P window of the scintillation counter for 1 min.
9. Regenerate the Dowex columns for reuse by adding 2 ml 1 M HCl.

3.3.6. Calculations

1. The specific activity for each assay is calculated from the radioactivity input taken as outlined in **Subheading 3.3.5, step 4**. The counts per min (cpm) are first converted to disintegrations per min (dpm) according to the efficiency of the counter. Using the 2.22×10^{12} dpm/Ci conversion, the dpms are converted to Ci. Since each reaction contains 100 µM ATP, the specific activity in Ci/mol is easily calculated.

2. The duplicate samples in the assay are averaged and the counts from the blank samples (as outlined in **Subheading 3.3.3, step 8**) are subtracted. The cpms are converted to dpms, to Ci, and to pmol as outlined in **step 1**. The pmol can then be converted to pmol/min/mg protein by dividing by the number of min the reaction ran for and by the amount of mg of protein in each sample, which can be obtained using a simple Bradford assay. Examples of data generated from receptor stimulation and MnSO_4^- and GTP γ S-mediated assays are given in **Fig. 1**.

4. Notes

1. To allow for maximal air exchange, the volume of media in a 250-ml flask should be kept to 100 ml.
2. When melting an agar suspension do not allow to boil as this will caramelize the agar and lead to experimental variability.
3. Cells for DB agar development are often plated at lower densities to test how robust the cells are in forming aggregates and fruiting bodies.
4. After the first aspiration the dishes are placed in a tilted position, where they are allowed to drain an additional 5 min, the remaining buffer is aspirated from the bottom.
5. The object here and in **Subheading 3.1, step 8** is to reduce excess fluid on the plate, which can lead to aberrant development. Allowing the cells to dry too long will also lead to aberrant development.
6. The number of cells to set up for pulsing depends on the assay performed. To avoid excess dilution with the repetitive pulsing, a minimum of 1×10^8 cells in 5 ml DB in a 75-ml Erlenmeyer flask is used.
7. Allow the cells to shake in the flask for 1 h prior to pulsing. This induces the expression of phosphodiesterases so that when cAMP is repeatedly added it is rapidly degraded. To pulse the cells we use a peristaltic pump (miniplus3, Gilson, Middleton, WI) connected to a timer (Chronrol XT, Chronrol Corporation, San Diego, CA) to turn the pump on and off every 6 min. The speed of the pump is calibrated to deliver 100 μ l of cAMP solution that will equal 75 nM when added to the flask each time the pump is activated. The tubing from the pumps is placed into flasks on an orbital shaker with variable speed. The pumps, orbital shaker, flasks, and controller are all housed inside a 22°C incubator.

8. Higher-resolution blots of cAR1 can be achieved by loading 45 μ l of prepared sample to a 12 well 10% Tris-HCl gel. This allows the phosphorylated and nonphosphorylated forms of the receptor to be visualized.
9. Both the ACA and the cAR1 antibodies are rabbit polyclonal antibodies. The cAR1 antibody needs to be precleared with fixed AX3 vegetative cells prior to use. Once diluted the antibodies are stored at 4°C in 3 mM sodium azide.
10. The membrane can be cut at the 100-kDa marker after the gel has transferred so that ACA on the upper fragment and cAR1 on the lower fragment can be blotted from the same gel.
11. A fluorescent dye such as rhodamine, which has a similar molecular weight as cAMP, can be loaded in the micropipette, visualized with appropriate fluorescent filters, and used to standardize the gradient emanating from the micropipette.
12. This step is very important because unpolymerized Dowex, which can cause large errors in the amounts of cAMP captured by the Dowex columns, needs to be removed. To do so, dH₂O is added to Dowex 1:1 v/v in a graduated cylinder, shaken, and the suspension is allowed to settle. The dH₂O is decanted and the wash is repeated three more times. We typically place the washed 1:1 Dowex/dH₂O slurry in a large beaker with a stir bar and gently stir the slurry overnight at 4°C. Afterward, the liquid is decanted away and the Dowex is washed two more times with dH₂O in a graduated cylinder to get rid of all remaining unpolymerized Dowex.
13. It is important to use the cells for the assay as soon as possible. We have detected fairly steep fall off rates for adenylyl cyclase activity after 1-hr incubation in ice.
14. Various concentrations of cAMP can be used depending on what is being investigated. We typically use saturating conditions where we stimulate cells with 10 μ M cAMP.

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Chapter 8

Quantitative Analysis of Distal Tip Cell Migration in *C. elegans*

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Summary

Correct distal tip cell (DTC) migration in the nematode *C. elegans* requires sensing soluble and matrix cues, remodeling extracellular matrix, and signaling through conserved integrin and netrin pathways. The DTC executes a complex path and coordinates its migration with the developmental stages of larval morphogenesis. This chapter outlines a method for investigating DTC migration in *C. elegans* using feeding RNA interference (RNAi) and light microscopy. To deplete a candidate gene of interest, nematode eggs are added to plates seeded with RNAi-inducing bacterial lawns. The animals hatch and begin to eat the RNAi bacteria, releasing dsRNA and causing the targeted gene to be depleted during larval development. Positions of migratory cells are monitored in larvae and young adults using differential interference contrast (DIC) and epifluorescence microscopy.

Key words: DTC, Cell migration, *C. elegans*, RNAi, DIC microscopy, Fluorescence microscopy

1. Introduction

Cell migration is of fundamental biological importance, essential for embryonic development and tissue and organ morphogenesis, and for immune system function and wound healing in the adult. Normal mechanisms of cell migration are hijacked by cancer cells during metastasis (1). Cell migration requires dynamic changes in the actin cytoskeleton, alterations in cell–cell and cell–extracellular matrix (ECM) contacts, and localized secretion of proteases (2). Mechanisms of cell migration, including regulation of integrin-containing adhesion complexes, Rac family GTPases, and the acto-myosin contractile machinery, are conserved throughout evolution (3). Quantitative investigation of cell migration in vivo

in the soil nematode *Caenorhabditis elegans* (*C. elegans*) allows gene discovery and analysis of gene function in cell migration.

C. elegans is an ideal model system with which to dissect the molecular basis of cell migration. The invariant pattern of cell migration, translucent body, and availability of cell specific GFP markers enable analysis of individual cells in the living animal. In addition, simple RNAi methodology, and a large and freely available collection of mutant strains greatly facilitate the study of *C. elegans* (4). Studies of axon guidance (5), sex myoblast migration (6), and distal tip cell migration (DTC) (3, 7, 8) have all helped to reveal the mechanisms by which migrating cells adhere to ECM molecules, interpret guidance cues, and coordinate cell movements with embryonic and larval stages. Examples of genes controlling many cell migratory events are listed in Table 1. Future studies of *C. elegans* will help elucidate how migrating cells interpret information, and how cells integrate multiple inputs and coordinate cytoskeletal and signaling proteins to influence cell polarity and generate directed cell motility.

Although many migratory cell types can be observed in *C. elegans*, this chapter will focus on the analysis of DTC migration. Gonad morphogenesis is dependent on migration of a specialized gonadal leader cell, the DTC (3). During the four stages of larval development (L1, L2, L3, and L4), the DTC radically changes direction exactly two times in response to attractive and repulsive cues to properly form the mirror image U-shaped gonad (3). At 23°C, it takes approximately 48 h for the DTC to migrate to its final location. The two DTCs are easily visible in the living worm, and the shape of each gonad arm reflects the migratory path taken by the DTC during larval development. If the DTC fails to migrate or follows an aberrant path, malformation of the gonad arm will result (Fig. 1).

In order to analyze the mechanism of DTC migration, candidate genes are targeted by RNAi and the effect on the animal monitored by light microscopy. RNAi is an effective method for analyzing gene function in *C. elegans* that often phenocopies loss-of-function phenotypes (9). In RNAi, double-stranded RNA (dsRNA) introduced into larvae or adults activates an enzymatic pathway that eliminates endogenous RNAs homologous to the dsRNA (10). Potent and persistent RNAi silencing in *C. elegans* results from secondary amplification of small amounts of the initial RNAi trigger by RNA-dependent RNA polymerases (11, 12). RNAi is commonly induced in *C. elegans* by injecting in vitro synthesized dsRNA into the body cavity of the animal, through creation of transgenic animals that express dsRNA from DNA arrays maintained within cells, or by feeding animals bacteria engineered to express dsRNA (feeding RNAi) (13, 14). The DTC is quite sensitive to gene knockdown by feeding RNAi. To demonstrate the response of the DTC to RNAi, we depleted

Table 1
Selected genes useful for cell migration studies in *C. elegans*

Locus	Gene ID	Function	Expression pattern	GFP line	Reference
<i>unc-6</i>	F41C6.1	Netrin	Intestine, muscle, and many migrating neurons	BC13558	(23)
<i>blh-8</i>	C02B8.4	<i>C. elegans</i> twist	Sex myoblast (SM) cells	PD4666	(24)
<i>unc-5</i>	B0273.4	Netrin receptor	Excretory cell (EXC), DTC, and Q cell descendant neurons	evIs98	(25)
<i>unc-73</i>	F55C7.7	Trio GEF	Hypodermis, muscle, DTC, EXC, Q cells, and migratory neurons	evIs80	(26)
<i>mig-10</i>	F10E9.6	Src homology (SH) and Plakstrin homology (PH), domains	Neurons (AVM, PVM, ALM, and CAN), pharynx, hypodermis, muscle	mpIs1	(27)
<i>ina-1</i>	F54G8.3	alpha integrin	DTC and gonad, Touch neurons, Q descendants, CAN, ALM, HSN	NG2517	(28)
<i>mig-2</i>	C35C5.4	Rac GTPase	Hypodermis, gonad, DTC, CAN, ALM, HSN, Q cells	CF579	(29)
<i>mig-13</i>	F43C9.4	Novel transmembrane protein	Hypodermis, intestinal-pharynx junction, neurons	muIs37	(30)
<i>gon-1</i>	F25H8.3	Matrix metalloproteinase (MMP)	DTC, male linker cells, muscles	BC13313	(23)
<i>unc-53</i>	F45E10.1	human NAV1, NAV2/RAINB1	EXC and pioneer axons	hdIs1	(31)
<i>mig-24/blh-12</i>	C28C12.8	Helix-loop-helix protein	DTC	nsIs65	(32)
<i>sax-3</i>	ZK377.2	<i>C. elegans</i> roundabout (ROBO)	Muscle, hypodermis, and all neurons	IC450	(33)
<i>ceb-23</i>	ZK652.5	Homeodomain protein	AIY, BAG, ASI, ADL, AWC, ASE, AFD, ASH, ASG, PHX, and CAN	NG2501	(34)
<i>lag-2</i>	Y73C8B.4	Transmembrane protein of the Delta/Serrate/Lag-2 (DSL) family	DTC	JK2533	(35)

(continued)

Table 1
(continued)

Locus	Gene ID	Function	Expression pattern	GFP line	Reference
<i>mec-4</i>	T01C8.7	Amiloride-sensitive Na ⁺ channel protein (degenerin)	Six touch neurons	SK4005	(36)
<i>unc-119</i>	M142.1	Novel	Pan-neuronal marker	DP132	(37)

The first and the second columns list the locus name and the gene ID for the gene of interest, followed by brief homology information in the third column. Cells and tissues expressing each gene are listed in the fourth column. In the fifth column, nonitalic alphanumeric strain names are available from the CGC (Caenorhabditis Genetics Center, Minneapolis, MN), whereas lowercase italic strains may be available from the researchers directly. Reference information is listed in the final column

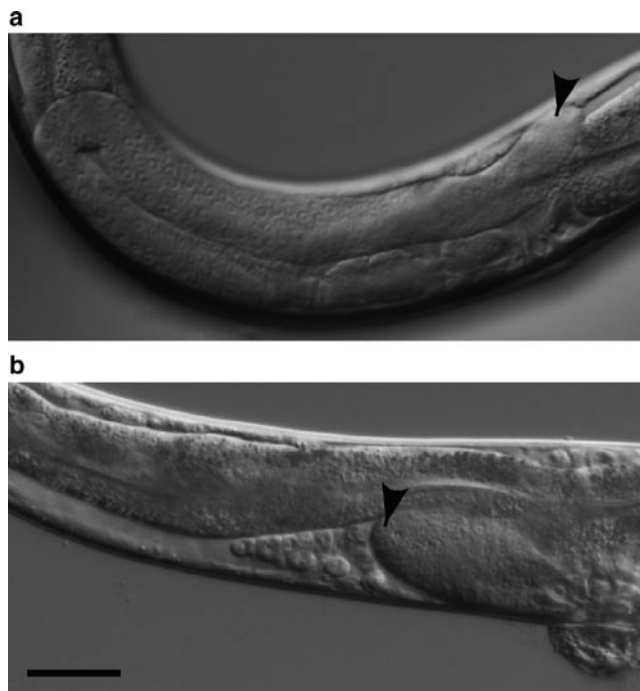


Fig. 1. Visualizing DTC migration defects with Nomarski microscopy. (a) Normal DTC migration results in a U-shaped gonad arm. Only the anterior gonad arm is shown. (b) In *gon-1/ADAMTS* RNAi the DTC does not migrate, resulting in a short gonad arm. Each DTC is indicated by an arrowhead. Magnification $\times 20$. Scale bar 20 μm .

DTC-expressed GFP by feeding RNAi and observed the level of GFP in the DTC after 48 h (Fig. 2).

The following sections describe a method for investigating cell migration in *C. elegans* using gene knockdown by feeding

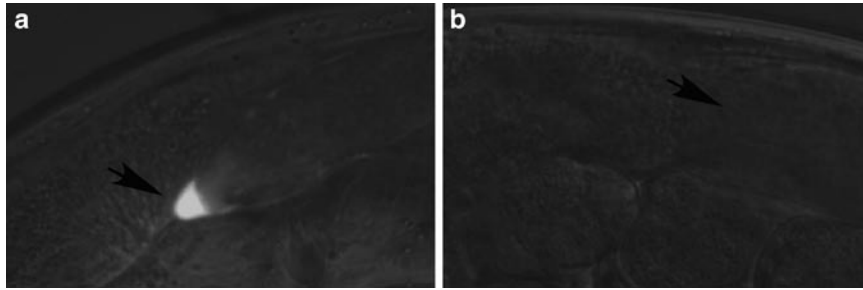


Fig. 2. Effective depletion of GFP in the DTC. *lag-2::GFP* animals were treated with (a) control or (b) GFP feeding RNAi. Each DTC is indicated by an *arrow*. Both panels are photographed under identical conditions; exposure time: 0.5 s. Magnification: $\times 40$.

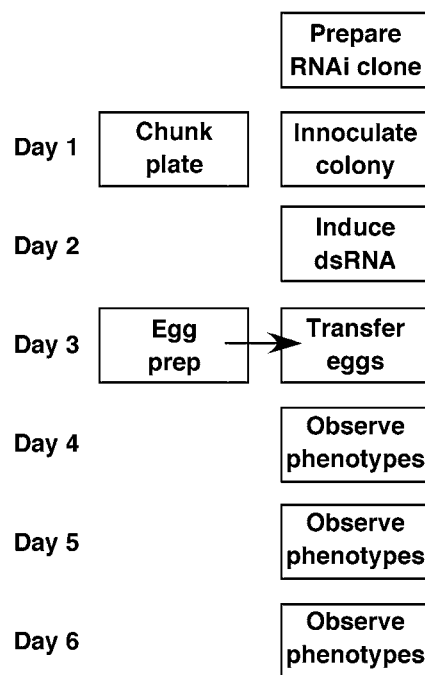


Fig. 3. Experimental timeline for the investigation of cell migration in *C. elegans* using RNAi.

RNAi and DIC microscopy (see timeline in Fig. 3). Briefly, the RNAi construct is transformed into bacteria optimized for the expression of dsRNA, the dsRNA is induced overnight on IPTG plates, and nematode eggs are added to the RNAi bacterial lawns. The animals hatch and begin to eat the RNAi bacteria, releasing dsRNA and causing the targeted gene to be depleted during larval development. Larvae and young adults are imaged using DIC or epifluorescence microscopy to monitor the position of the migratory cells.

2. Materials

2.1. Obtaining a Construct for Feeding RNAi

1. RNAi feeding vector pPD129.36, also known as L4440 (AddGene, Cambridge, MA) (*see* [Note 1](#)).
2. HT115 (DE3) bacteria optimized for the production of dsRNA (Caenorhabditis Genetics Center, Minneapolis, MN) (*see* [Note 2](#)).
3. Z-Competent *E. coli* transformation kit (Zymo Research, Orange, CA) (*see* [Note 3](#)).
4. RNAi clone targeting your gene of interest from the Ahringer RNAi library ([15](#)) (Geneservice, Ltd. Nottingham, UK) or the Vidal RNAi library ([16](#)) (Open Biosystems, Huntsville, AL).

2.2. Feeding RNAi

1. Nematode growth media (NGM) plates with IPTG and carbenicillin (NGM-IPTG): 0.3% (w/v) NaCl, 1.7% (w/v) Bacto agar, 0.25% peptone (Fisher). Autoclave and, after the plate mix cools to 55°C, add 1 mL of 5 mg/mL cholesterol in ethanol. Then add the following components, using filter-sterilized stock solutions, to the indicated final concentrations: 1 mM CaCl₂; 1 mM MgSO₄; 40 mM potassium phosphate buffer, pH 6; 40 mg/L carbenicillin disodium salt (Fisher); 100 mg/L isopropyl β-d-thiogalactoside (IPTG) (Ambion, Austin, TX); and 5 mL/L nystatin suspension (optional) (Fisher) (*see* [Notes 4](#) and [5](#)).
2. LB-Amp: 25 g LB mix (Fisher) per liter, autoclave and add 40 μg/mL ampicillin after solution cools, store at 4°C. For LB-Amp plates add 15 g Bacto agar per liter to the LB mix before autoclaving.
3. *C. elegans* (Caenorhabditis Genome Center, Minneapolis, MN) (*see* [Note 6](#)).

2.3. Preparing *C. elegans* Embryos

1. NGM plates (as described in **item 1** of **Subheading 2.2** except lacking IPTG and carbenicillin) seeded with OP50 *E. coli* (Caenorhabditis Genome Center, Minneapolis, MN) for propagation of nematodes.
2. Bleach solution: 2 mL household bleach, 4.5 mL H₂O, 0.5 mL 10N NaOH. Bleach solution should be made fresh.
3. M9: 22 mM KH₂PO₄, 22 mM Na₂HPO₄, 86 mM NaCl, 1 mM MgSO₄. Filter sterilize.

2.4. DIC Microscopy

1. Microscope cover slips (22 × 22 × 0.15 mm) and VistaVision Microscope slides (3 in. × 1 in. × 1 mm) (VWR) (*see* [Note 7](#)).
2. 2% agarose (Fisher) in H₂O, microwaved to melt.

3. 80 mM sodium azide in M9 (Arcos organics) (this is a neurotoxin, so care should be taken to avoid exposure) (*see* [Note 8](#)).
4. Worm pick: Gauge 28 platinum wire (VWR or Tritech Research, Los Angeles, CA), melted to the end of a Pasteur pipet.

3. Methods

One of the main advantages of using *C. elegans* to study cell migration in vivo is the facility with which gene function can be disrupted using RNAi. RNAi is an effective method for analyzing gene function that often phenocopies loss-of-function phenotypes ([9](#)). In RNAi, dsRNA introduced into larvae or adults activates an enzymatic pathway that eliminates endogenous RNAs homologous to the dsRNA ([10](#)). This feeding RNAi protocol is slightly modified from ([17](#)) and can be used for candidate gene or broad RNAi screens in *C. elegans* ([8, 18](#)). Another important advantage of the *C. elegans* system is that the animal's body is transparent, so cells can be viewed in the living animal using DIC microscopy. Transgenic lines expressing GFP can be used to facilitate identification and positioning of migratory cells ([Table 1](#)).

The DTCs follow a stereotypical migratory path during larval development (*see* [Fig. 1](#)). The DTC begins to migrate in the larval stage L2. In mid L3, ~30 h after hatching, the DTCs make a 90° turn from the ventral surface of the body wall muscle and move dorsally across the lateral hypodermis. As the cells near the dorsal body wall, they quickly reorient and migrate at about 10 µm per hour until the L4/adult molt ([19](#)). The final shape of the gonad arm reflects the path taken by the DTC during larval development. In addition, the position and orientation of the DTC can be documented during each turn. Therefore, it is quite straightforward to knock down a gene of interest by feeding RNAi, and observe the effect on DTC migration in larval or adult animals using a live wet mount and light microscopy.

3.1. Obtaining a Construct for Feeding RNAi

1. Obtain RNAi constructs targeting your gene of interest (*see* [Subheading 2.1](#)) (*see* [Note 9](#)). Ahringer and Vidal library RNAi clones are supplied as HT115 (DE3) frozen bacterial stocks and are ready to use for feeding RNAi.
2. Obtain the *gon-1* RNAi (Ahringer library clone IV-5M07) construct for use as a positive control. RNAi to *gon-1* prevents DTC migration resulting in short, stubby gonads (*see* [Fig. 1](#)).
3. Prepare competent HT115 (DE3) cells using the Z-Competent kit (*see* [Note 3](#)).

4. Transform HT115 (DE3) bacteria with pPD129.36 vector only (no insert). In this negative control, dsRNA is synthesized from vector sequences that do not specifically target *C. elegans* transcripts. Streak transformed cells on LB-Amp plates to select colonies.

3.2. Feeding RNAi

1. Streak bacteria from each RNAi targeting construct (and controls) on an LB-Amp plate and grow overnight at 37°C.
2. Inoculate a single colony from each RNAi targeting construct (and control) into LB-Amp broth and grow in a shaking incubator overnight at 37°C (**Fig. 3**, Day 1).
3. Spot 150 μ L of each culture onto individual NGM-IPTG plates and induce dsRNA overnight at room temperature (*see Note 10*) (**Fig. 3**, Day 2).
4. Transfer eggs prepared from gravid nematodes (*see Subheading 3.3*) to RNAi food plates; incubate plates at 23°C (**Fig. 3**, Day 3).

3.3. Preparing *C. elegans* Embryos

1. Allow *C. elegans* hermaphrodites (*see Note 6*) to grow on NGM plates seeded with OP50 bacteria until starved. Starved plates can be stored at 15°C for up to one month.
2. Cut a 1-cm square of agar from the starved plate using a spatula. Transfer the “chunk” to a fresh NGM plate seeded with OP50 bacteria, flipping the chunk upside down onto the edge of the OP50 lawn. Animals arrested as dauer larvae will emerge from the chunk and recommence larval development. Incubate the plate at 23°C for 2 days. The starved plate should be chunked on the same day that colonies are inoculated into LB-Amp (*see Subheading 3.2, step 2*) (**Fig. 3**, Day 1).
3. On Day 3, gently add 1 mL M9 to the NGM plate. Collect gravid hermaphrodites by tipping the plate slightly and pipetting the M9 into a snap cap tube.
4. Pellet the swimming animals by centrifuging for 30 s at 9,000 $\times g$. Remove the supernatant taking care not to disturb the loose pellet of worms.
5. Add 1 mL bleach solution and vortex. Incubate 5 min at room temperature with occasional vortexing (*see Note 11*). Pellet the eggs by centrifuging for 30 s at 9,000 $\times g$.
6. Remove the supernatant, and rinse the pellet with 1 mL M9. Pellet the eggs by centrifuging for 30 s at 9,000 $\times g$. Repeat.
7. Remove the supernatant, and resuspend the pellet in ~100 μ L M9. Vortex the sample. Quickly place a 2- μ L drop of eggs on a slide, and use a dissecting microscope to estimate the number of eggs per μ L in the sample.
8. Transfer ~200 eggs to the NGM-IPTG plates prepared in **Subheading 3.2, step 4** (*see Note 12*).

3.4. DIC Microscopy

As indicated by **Fig. 3**, DTC migration can be observed on day 4, day 5, or day 6 of the protocol. On day 4, the animals will be young larvae, with DTCs in the early stages of cell migration. On day 4 (~48 h after egg prep), the larvae will be L4s and the majority of the DTC migration program will be completed. On day 5, the animals will be young adults, and the final position of the DTC can be determined. As the hermaphrodites become older, they will begin to fill with embryos precluding accurate scoring of DTC migration.

1. Prepare a wet mount slide for DIC microscopy. Melt 2% agarose in H₂O by microwaving, and place a 1-cm diameter drop in the center of a microscope slide.
2. Quickly cover the drop with a coverslip to flatten it into an agar pad. Allow the agar pad to solidify (*see Note 13*).
3. Carefully remove the coverslip and add a 5- μ l drop of 80 mM sodium azide in M9 to the center of the pad.
4. Add larval or adult animals to the drop using the platinum pick, and gently replace the coverslip.
5. View slides under DIC microscopy, and locate the DTC. The position of the DTC is at the distal end of the gonad arm. The gonad arm can easily be identified as a thick tube filled with Cheerio[®]-shaped germ cells (*see Fig. 1*). If a GFP transgenic nematode has been used for the RNAi experiment, excitation at 490 nm (blue) induces the fluorescence at 509 nm (green). Software such as Spot Advanced can be used to overlay the DIC and fluorescence images (*see Note 14*).
6. Document the DTC migration paths in the experimental and control samples using photomicroscopy. If desired, the distance migrated by the DTC to each of the turns and total distance can be determined using software such as Spot Advanced (*see Fig. 4*).

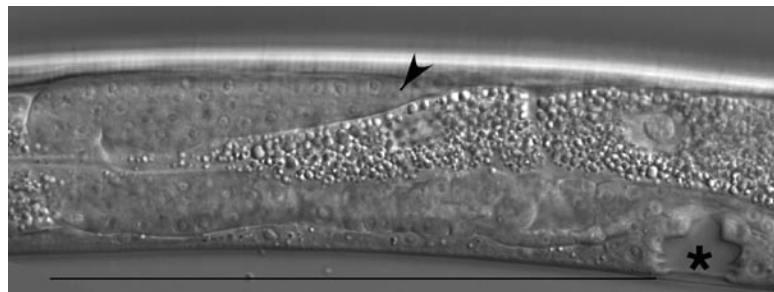


Fig. 4. Documenting DTC migration. The DTC in this L4 larva is indicated by an *arrowhead*. The developing vulva is marked by an *asterisk*. The distance migrated by the DTC (vulva to first turn) is indicated by the *black line* and equals 130 μ m. Magnification: $\times 60$.

4. Notes

1. If desired, cDNA of your gene of interest can be easily inserted into pPD129.36 using standard RT-PCR cloning. Inserts greater than 500 base pairs in length tend to be the most effective for RNAi in *C. elegans*.
2. HT115 (DE3) is not a very good strain for molecular cloning. A strain such as DH5 α is usually used for cloning steps. The final RNAi construct is then transformed into the HT115 (DE3) cells using the Z-Competent kit.
3. Although we find the Z-Competent kit to be by far the most effective, other protocols for making competent cells can be used with HT115 (DE3).
4. Some experience working with nematodes is assumed. For exact instructions on nematode husbandry and for making NGM plates see (4).
5. Alternatively, ampicillin and beta-lactose can be used instead of carbenicillin and IPTG.
6. Wild-type nematodes (N2) can be used for RNAi. If stronger knockdown is required, an RNAi-sensitive strain such as *rrf-3(pk1426)* strain NL2099 can be used. The *rrf-3(pk1426)* strain is temperature sensitive and sterile at 25°C. Transgenic strains expressing GFP in cell types of interest (*see* **Table 1**) are also available and facilitate identification of migratory cells.
7. Some members of our laboratories prefer 20 $\times$ 40 mm, 20 $\times$ 50 mm, or 20 $\times$ 64 mm coverslips (all available from VWR).
8. If it is desirable to recover living nematodes after microscopy, the animals can be anesthetized in 0.01% levamisole (Sigma), 0.1% tricaine (Sigma) in M9 instead of sodium azide.
9. If a positive result (effect on cell migration) is obtained with the selected RNAi targeting clone, it is important to construct several other nonoverlapping RNAi targeting constructs to demonstrate the phenotype is result of specific knockdown of the targeted gene (20).
10. Two sets of NGM-IPTG plates can be prepared so that induced plates are ready to test a second generation of nematodes.
11. The solution should become clear as the nematodes are dissolved by the bleach solution and mechanically disrupted by the vortexing. Do not allow bleaching to proceed beyond 6 min, because egg viability will decrease.
12. The egg prep partially synchronizes the nematodes to within about 7 h of the same developmental age. Nematode populations can be more closely synchronized by hatching eggs

in the absence of a bacterial food source. Upon transferring arrested L1s to food, larval development will commence (21). Alternatively, gravid adults can be allowed to lay eggs for 30 min and then removed. The resulting age-matched hatchlings can then be monitored throughout development.

13. It is important that the agarose pad be thin. This can be accomplished by preheating the slides on a 65°C heat block so that the agarose solution does not cool too quickly before the coverslip is placed on top. Do not attempt to flatten the agarose pad by pressing down on the coverslip.
14. Typically 50 individuals from each RNAi experiment are analyzed by DIC microscopy and the proportion of the population with faulty DTC migration compared using 95% confidence intervals for proportions (22).

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Chapter 9

Inflammation and Wound Healing in *Drosophila*

Brian Stramer and Will Wood

Summary

Cell motility is a widely researched and clinically relevant process that has primarily been investigated using cell culture models. While these in vitro assays are useful in allowing for high-resolution analysis of cell movement, there will always be questions surrounding the physiological relevance of studying cell migration on artificial 2-dimensional substrates. Therefore, a number of groups in recent years have started developing alternative systems, either ex vivo or in vivo, to begin extrapolating our knowledge surrounding cell motility to actual developmental and disease processes. One such example exploits the translucence of *Drosophila* embryos, and the genetic tractability of this well-characterized model organism, to understand the cellular and molecular events surrounding inflammation and wound healing. Laser ablation of a small patch of embryonic epithelium in the *Drosophila* embryo results in a repair process that can be timelapse imaged in its entirety as the epithelial hole is sealed shut. Additionally, *Drosophila* macrophages can be imaged as they rapidly respond and chemotax to these sites of damage in a process reminiscent of the vertebrate inflammatory response. In both cases the imaging is of a spatial and temporal resolution approaching that which can be obtained from in vitro systems, making the *Drosophila* embryo an ideal model to begin dissecting the genetic control of cell migration during wound healing and inflammation in an in vivo setting.

Key words: *Drosophila*, Hemocyte, cell migration, wound healing, timelapse microscopy, Gal4-UAS system

1. Introduction

The best way to understand cell migration during cell and tissue movements is to watch it in vivo using timelapse analysis, and the *Drosophila* embryo lends itself perfectly to such live imaging. *Drosophila* development will proceed normally at room temperature with embryos simply mounted on a slide without using complicated media or environmentally controlled chambers. Furthermore, embryos are translucent, and the powerful genetics

of this model organism allows for the rapid generation of animals containing Green Fluorescent Protein (GFP)-fusion proteins expressed in virtually any cell type or tissue of interest. Use of the Gal4-UAS system (1) has allowed for the *in vivo* examination of cell migration in a number of different *Drosophila* cell types during embryonic development, e.g., germ cells (2), epithelial cells (3), and tracheal cells (4), to name just a few.

Here we describe a detailed protocol for live imaging of inflammatory cell migration and repair processes in *Drosophila*. Laser ablation of stage 15 *Drosophila* embryos leads to immediate destruction of a circular patch of epidermis approximately ten cells in diameter. Using the Gal4-UAS system to drive expression of GFP-fusion proteins in the epithelium, the process of wound closure can be imaged live using timelapse confocal microscopy offering a dynamic dimension to the study of this clinically relevant process. These wounds reproducibly re-epithelialize in a few hours by a combination of actomyosin “purse-string” contraction and filopodial-driven adhesion of the epithelial edges in a process reminiscent of events observed during the repair of a number of different wounded tissues in vertebrates (Fig. 1a, b) (5, 6).

Furthermore, in response to the same epithelial laser damage an inflammatory process will ensue. In vertebrates, inflammation is an important aspect of tissue repair whereby white blood cells actively chemotax to sites of damage, where they phagocytose wound debris and bacteria that have penetrated the epithelial breach in order to prevent what is likely an increased risk of infection. Similarly, laser ablation of *Drosophila* embryos triggers a rapid chemotactic response from hemocytes (7), which are an analogous cell type to vertebrate leukocytes. Quantitative analysis reveals that the response is extremely fast, with the first hemocytes arriving within 5–10 min of the laser ablation event. The number of hemocytes at the wound site steadily increases with time until a peak at 1 h (Fig. 1c, d) (7). Subsequently, hemocyte numbers at the wound decline, allowing the resolution phase of the inflammatory response to also be modeled in this system. Expression of GFP in these cells using hemocyte-specific Gal4 lines reveals the presence of dynamic, polarized lamellipodia extending from the hemocyte leading edge as they chemotax toward the wound, showing that this system is amenable to high-resolution analysis of the migratory process (7).

We have also developed a mechanical wound assay whereby a heparin bead is placed into the wound site. Unlike the laser assay, this system allows for drug manipulation as the bead can be pre-treated with pharmacological agents that may modulate hemocyte migratory responses. Similar to laser-induced wounds, untreated bead implantation results in rapid accumulation of hemocytes (Fig. 1e), while pre-treatment of beads with inhibitors of known signaling pathways important for cell migration,

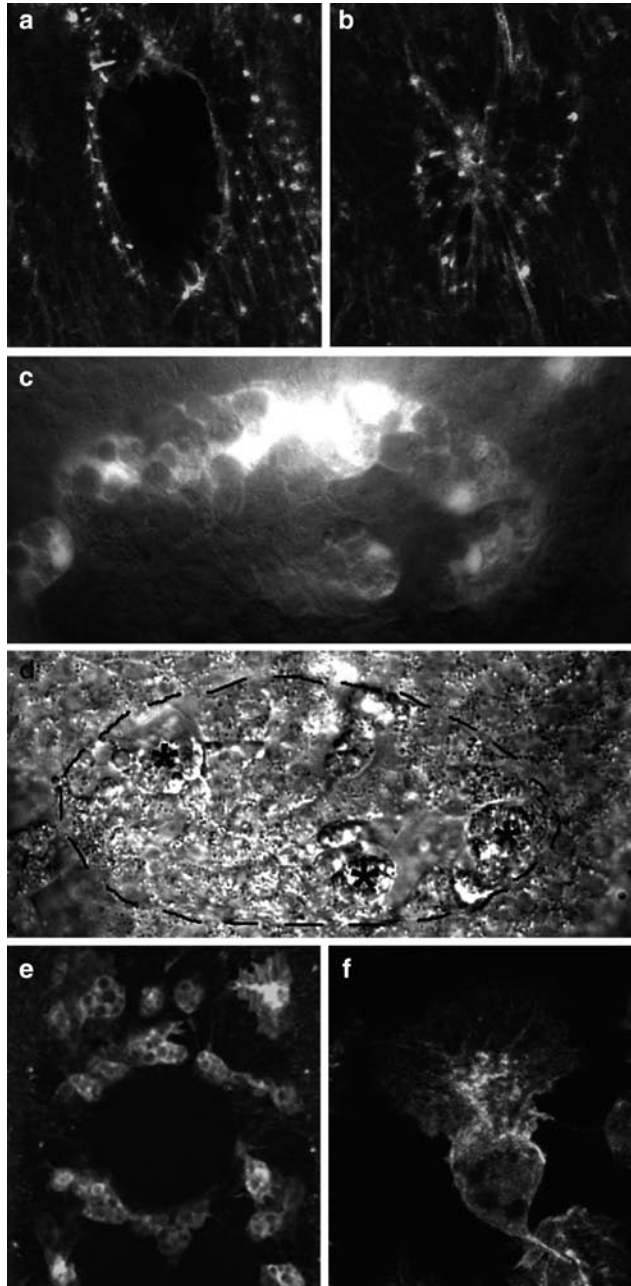


Fig. 1. Wound healing and inflammation in *Drosophila* embryos. (a) A laser ablation wound to a moesinGFP expressing *Drosophila* embryo will, by a “purse-string” mechanism, (b) seal shut over a period of a few hours. (c) This same laser wound will also recruit hemocytes that, by driving GFP specifically in these cells, can be imaged by widefield microscopy. (d) Alternatively, the wound site can be imaged by Differential Interference Contrast microscopy to give a general idea of wound size (*dotted line*) and hemocyte presence (*). (e) The insertion of a heparin bead into the wound site will also lead to the recruitment of hemocytes that will surround and encapsulate the bead. (f) A hemocyte undergoing developmental dispersal viewed by confocal microscopy.

such as phosphoinositide 3-kinase (PI3K), inhibits the process (8). The addition of drug manipulability to an already genetically tractable model greatly increases the experimental scope of this migration assay.

Embryonic hemocytes carry out many important functions during development (9). In order to arrive at their correct location to carry out these tasks, hemocytes must undergo long-range migration as they leave their developmental origin in the head and distribute themselves evenly throughout the animal. This developmental dispersal is a tightly controlled, programmed event with hemocytes following a stereotypical route. One major “highway” is along the nerve cord in the ventral midline. Live imaging of hemocytes along this structure has revealed that a subset will leave the midline and migrate laterally at specific “exit points” (8). During this lateral movement hemocytes will increase their speed and directionality, and display highly polarized lamellipodia similarly observed when these cells are migrating to wounds (Fig. 1f). The advantage of this developmental migration assay over the aforementioned models is that cells chemotax over greater distances, allowing for longer cell tracking and therefore a more detailed analysis of cell motility.

Here we describe a series of methods that will enable the researcher to make epithelial wounds in *Drosophila* embryos by either laser ablation or bead implantation, and to image the resulting wound healing and inflammatory responses in real time. These same methods can be used to image the normal developmental migration of hemocytes. While this chapter will introduce the most basic of fluorescent constructs that allow for gross analysis of cellular motility in these cell types, a number of readily available fluorescent probes can be used to analyze finer intracellular detail to further dissect the migration process. This system is ideal for the cell biologist interested in motility, as the cellular movements outlined here can be imaged to a very high spatial and temporal resolution allowing for a detailed in vivo dissection of cell migration.

2. Materials

2.1. Embryo Collection

1. Egg laying cages (Fig. 2d).
2. Apple juice agar plates (Fig. 2e).
3. Yeast.
4. Fine Paintbrush.
5. Commercial bleach (e.g., Clorox).

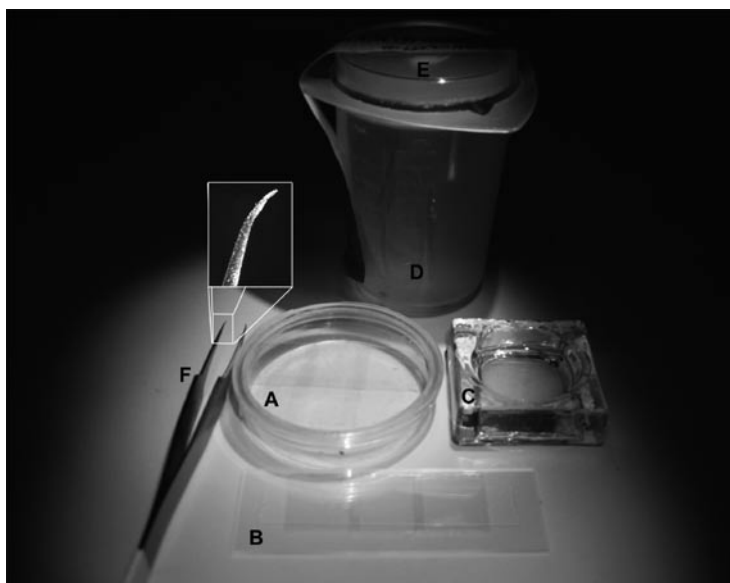


Fig. 2. Critical tools required for embryo collection and mounting. (a) An example of the *Method A* mounting procedure, which requires a gas-permeable culture dish with embryos mounted on the underside in halocarbon oil beneath a coverslip. (b) An example of the *Method B* mounting procedure, which involves sticking the embryos down to a microscope slide with double-sided sticky tape, and covering with halocarbon oil and a coverslip. (c) The watch glass used for dechorionation and selection of embryos. (d) The laying cage is simply a plastic beaker covered by (e) an apple juice agar plate where flies lay their eggs. (f) The most critical utensil for embryo mounting is the “scooper,” which is forceps with a very slight bend at the tip of one of the tines.

6. Watch glass (Fig. 2c).

7. Petri dish.

8. H₂O.

9. Dissecting scope.

2.2. Embryo Mounting and Culture

2.2.1. Method A (see Fig. 2a)

1. “Scooper” – Thin forceps with a slight bend at the tip (Fig. 2f) (see Note 1).
2. Greiner Lumox gas-permeable culture dish (previously known as petriPERM, Sigma).
3. Halocarbon oil (mixture of 50% halocarbon 27 and 50% halocarbon 700, Sigma).
4. 18 × 18mm coverslips #1 thickness.
5. Nail polish.
6. Dissecting scope.

2.2.2. Method B (see Fig. 2b)

1. “Scooper.”
2. Microscope slides.

3. Double-sided sticky tape (Scotch 3MM).
4. Halocarbon oil (mixture of 50% halocarbon 27 and 50% halocarbon 700, Sigma).
5. 18 × 18mm coverslips #1 thickness.
6. Nail polish.
7. Dissecting scope.

2.3. Epithelial Wound Assay

1. Micropoint Ablation Laser (Photonics Instruments).
2. Fluorescent *Drosophila* lines:
 - (a) Epithelial Gal4 driver lines, e.g., e22c-Gal4 ([1](#)).
 - (b) Fluorescent UAS lines, e.g., UAS-moesinGFP ([10](#)), which labels filamentous actin. UAS-actinGFP can also be used ([11](#)) (*see* [Note 2](#)).

2.4. Hemocyte Migration Assays

2.4.1. Hemocyte Developmental Dispersal

1. Fluorescent *Drosophila* lines:
 - (a) Hemocyte Gal4 driver lines, e.g., srp-Gal4 ([12](#)). This driver line is expressed from stage 11 onward and is therefore the best for imaging early hemocyte movements.
 - (b) Fluorescent UAS lines, e.g., UAS-moesinGFP ([10](#)).

2.4.2. Hemocyte Wound Chemotaxis

1. Micropoint Ablation Laser (Photonics Instruments).
2. Fluorescent *Drosophila* lines:
 - (a) Hemocyte Gal4 driver lines, e.g., srp-Gal4 ([12](#)), pxn-gal4 ([7](#)) (expressed from stage 12 onwards), or crq-gal4 ([7](#)) (expressed from stage 12 onwards).
 - (b) Fluorescent UAS lines, e.g., UAS-moesin-GFP ([10](#)), or UAS-GFP.

2.4.3. Hemocyte Bead Chemotaxis

1. Transgenic fly lines as outlined in **Subheading 2.4.1** covering hemocyte developmental dispersal (*see* **Subheading 2.4.1**).
2. Sharpened tungsten needle.
3. 1.5ml microfuge tubes.
4. Heparin beads (Sigma) or Affigel blue beads (Bio-Rad Laboratories).
5. Dessicator.
6. Forceps.

2.5. Postmicroscopy Editing

1. ImageJ software. Download from <http://rsbweb.nih.gov/ij/>.
2. *ImageJ software plugins*. From the same website, also download appropriate input plugin to open image series (depends on software utilized to acquire timelapse series), Group_ZProjector, and concatenator.

3. Methods

3.1. Embryo Collection

1. Incubate appropriate fly lines (*see Note 3*) in a laying cage with a base comprising a Petri dish filled with apple juice agar, supplemented with a pea size drop of yeast paste (yeast paste consists of dried yeast mixed with H₂O to the consistency of peanut butter).
2. Change the apple juice agar plates every 1–2 h and age appropriately to obtain embryos at the required developmental stage. Stage 15 embryos should be used for epithelial wound and hemocyte wound assays, which requires aging embryo collections overnight at 18°C. Slightly younger embryos (stages 13–14) should be used for imaging hemocyte developmental dispersal (*see Note 4*).
3. Using a paintbrush, transfer the embryos from the apple juice agar plates to a watch glass filled with commercial bleach.
4. Observe the embryos under a dissecting microscope. Once the embryos have burst from their chorion, using forceps collect the correct stage embryos and move to a drop of water on a clean Petri dish. To aid in the collection you may want to very carefully bend the end of the forceps to create a “scooper” to pick up embryos from the watch glass. Ensure that the embryos do not stay in the bleach for more than 5 min.
5. Using forceps transfer embryos for appropriate mounting.

3.2. Embryo Mounting and Culture

We provide two different methods for the mounting of embryos. Although the overall approach is the same in each, the two methods are suited to different experiments depending on the length of imaging time. *Method B* is more suitable for shorter timelapse movies of less than 3 h, whereas *Method A* is recommended for longer timelapse sessions. Additionally, when the orientation of an embryo is crucial to the experiment such as during imaging of hemocyte developmental dispersal, *Method B* is recommended. This is due to the fact that since embryos are stuck down with this method they are less likely to roll out of position when the coverslip is placed over them.

3.2.1. Method A

1. On the bottom of an inverted gas-permeable culture dish place two coverslips on either side of the dish.
2. Using forceps remove the embryos from the drop of water and gently transfer onto a drop of Halocarbon oil. This allows for easier transfer to the culture dish.
3. Move the embryos from the drop of halocarbon oil to the center of the culture dish in between the coverslips (*see Note 5*).

4. Line up embryos between the coverslips.
5. Cover the embryo with a small drop of Halocarbon oil (*see* **Note 6**).
6. Cover the embryos with a coverslip “bridge.” The two coverslips on either side of the embryos act as supports for the “bridge” creating a small oil chamber where the embryos will develop normally.
7. Glue the coverslips in place using a small amount of nail polish carefully applied to the points of overlap between coverslips.

3.2.2. Method B

1. Cover a glass slide with 3 cm of double-sided sticky tape.
2. Using forceps remove the embryo from the drop of water and gently place on the sticky tape.
3. Roll the embryo gently with forceps to precisely position it for live imaging (i.e., ventral side up for imaging hemocyte migration along the developing nerve cord). This positioning should be done quickly since the embryo rapidly dries out in only a few minutes if not covered in oil.
4. Cover the embryo with a small drop of Halocarbon oil (*see* **Note 6**).
5. Place a small coverslip either side of the embryo at a distance of approximately 2 cm and “bridge” with a second coverslip.
6. Glue the coverslips in place using a small amount of nail varnish carefully applied to the points of overlap between coverslips.

3.3. Epithelial Wound Assay

1. Collect e22c-Gal4 X UAS-moesinGFP embryos as explained in **Subheading 3.1**.
2. Change the apple juice agar plates every 1–2 h and age appropriately to obtain embryos at the required developmental stage. Stage 15 embryos should be used for epithelial wound and hemocyte wound assays, which requires aging embryo collections overnight at 18°C.
3. Dechorionate embryos and place in a drop of H₂O as explained in **Subheading 3.1**. Mount embryos using *Method A* and try to orient them with their ventral sides facing up. This does not have to be perfect.
4. Using a 40× or 63× objective, ablate a small patch of epithelium with the ablation laser. The hole generated should be approximately 40–50 μm in diameter (*see* **Note 7**).
5. Timelapse image epithelial wound closure by confocal microscopy. The Z-stack should be large enough to observe the entirety of the epithelium which will be approximately 2–3 μm thick. One minute between images is sufficient to quantify wound closure.

6. As the wound closes, the epithelium will be pulled down by the actin “purse string”, which will require minor adjustments in the focus (*see* **Note 8**).

3.4. Hemocyte Migration Assays

3.4.1. Hemocyte Developmental Dispersal

1. Collect srp-Gal4 X UAS-moesinGFP embryos as explained in **Subheading 3.1**.
2. Change the apple juice-agar plates every 1–2 h and incubate at 18°C for 16 h in order to obtain stage 13–14 embryos suitable for imaging hemocyte migration on the developing ventral nerve cord.
3. Collect embryos and mount as described in **Subheading 3.1**, orienting embryos ventral side up using either *Method A* or *Method B*. While either method can be used we would recommend using *Method B* since it is crucial to position embryos correctly in order to achieve the best images.
4. Image hemocyte dispersal by confocal microscopy. Movies should be started when a single row of hemocytes are lined up along the ventral midline. The Z-stack should be approximately 3–4 µm thick and images should be taken approximately every 30–60 s.

3.4.2. Hemocyte Wound Chemotaxis

1. Collect srp-Gal4 X UAS-moesinGFP embryos as explained in **Subheading 3.1**.
2. Change the apple juice agar plates every 1–2 h and age appropriately to obtain embryos at the required developmental stage. Stage 15 embryos, which contain evenly dispersed hemocytes, should be used for wound assays.
3. Dechorionate embryos and place in a drop of H₂O as explained in **Subheading 3.1**. Mount embryos ventral side up using *Method A*.
4. Using a 40× or 63× objective, ablate a small patch of epithelium with the ablation laser. The hole generated should be approximately 40–50 µm in diameter and will recruit, on average, 8–9 hemocytes (*see* **Note 9**).
5. Timelapse movies can be taken on a widefield scope for basic quantification of hemocyte recruitment to wounds. For higher resolution of hemocyte morphology confocal microscopy should be used as in **Subheading 3.4.1**.
6. To simultaneously examine epithelial closure and hemocyte recruitment Differential Interference Contrast (DIC) microscopy can be used, which will give a general wound outline.

3.4.3. Hemocyte Bead Chemotaxis

1. Collect srp-Gal4 X UAS-moesinGFP embryos as explained in **Subheading 3.1**.

2. Change the apple juice agar plates every 1–2 h and age appropriately to obtain embryos at the required developmental stage. Stage 15 embryos, which contain evenly dispersed hemocytes, should be used.
3. Presoak either heparin beads or Affigel blue beads in chosen drug for 3 h prior to mounting embryos. This can be done by incubating the beads at 4°C in a small drop (3 µl) of drug solution pipetted onto the inner concave surface of a 1.5 ml microfuge tube cut in half lengthways.
4. Collect embryos as described in **Subheading 3.1** and follow the *Method B* mounting instructions.
5. Once embryos are sitting on double-sided sticky tape, position embryos such that their ventral side is facing up.
6. Transfer the slide to a dessicator for 6 min to dehydrate the embryos.
7. Remove slide from dessicator and cover embryos with Halocarbon oil.
8. Using forceps or a sharpened tungsten needle transfer the drug-soaked beads into the Halocarbon oil and position the bead on the uppermost surface of the embryo.
9. Very carefully, using a sharpened tungsten needle make a small incision in the vitelline membrane and pierce the epithelium of the embryo.
10. Position the bead above this incision and gently push to implant into the underlying epithelial incision.
11. Continue mounting as described in **Subheading 3.2.2, steps 5–6**. As the rectangular bridging coverslip is placed over the embryo its weight will help to force the bead into the embryo.
12. For basic quantification, imaging can be carried out on a widefield microscope, but for better resolution and accurate quantification we recommend imaging using a confocal microscope as outlined in **Subheading 3.4.1**.

3.5. Postmicroscopy Editing

Image processing of the timelapse series can be done using either proprietary software or ImageJ (<http://rsb.info.nih.gov/ij/>), which is an open-source image processing program written in Java. This program can be used as a very basic tool to open and compile movies as well as for more complicated analysis and quantification. Only a very introductory tutorial on movie editing will be discussed here.

1. Open timelapse series in ImageJ. This may require an additional plugin from the ImageJ website, depending on the microscopy software used to acquire the timelapse series.

2. Use the “Group_Zprojector” plugin to flatten each Z stack series into single frames.
3. For long movies, where more than one movie sequence needs to be spliced, use the “concatenator” plugin to join the two series.
4. Depending on the quality of movie, various digital filter plugins can be used in ImageJ to “clean-up” the final movie sequence.
5. For basic analysis of hemocyte chemotaxis, the “manual tracking” plugin in ImageJ can be used.
6. The final movie series can be saved as a .tif file, which is recommended if additional analysis is required. Alternatively, the image sequence can be exported as a movie file (e.g., .avi format), for presentation or publication purposes.
7. More complex cellular analyses, such as kymography, which are commonly used for in vitro models, are also possible using these in vivo models of cell migration. Furthermore, we have only dealt with the analysis of gross cellular movements; however, the dynamics of intracellular components important for cell migration (e.g., actin and microtubules) can also be examined in this system (*see* **Note 10**).

4. Notes

1. A good “scooper” is a critical piece of equipment for this assay as it will greatly aid in the collection of properly staged embryos. We unfortunately have no good advice on how to generate the optimal bend in the tip of the forceps and simply recommend trial and error.
2. UAS-actinGFP can be toxic as cellular movements can be slightly disrupted when actin is overexpressed.
3. We have only introduced very basic GFP lines in this review. There are now a host of different fluorescent transgenic fly lines that can be used to label a variety of intracellular organelles (e.g., golgi, mitochondria), cellular compartments (e.g., membrane, nucleus), or cytoskeletal components (e.g., microtubules) that one may choose to utilize depending on the research question.
4. If weaker GFP lines are used we recommend not changing the apple juice agar plates and keeping the laying cages at 25°C overnight. Use the overnight plate for embryo collection. While this will make embryo collection a little more difficult, Gal4 is temperature sensitive and embryos will therefore be brighter for imaging.

5. To our knowledge the only commercially available gas-permeable culture dishes are unfortunately hydrophilic, which results in embryos sliding around during the mounting procedure. However, as these dishes can be reused, with time this problem will dissipate as the hydrophilic coating is lost. To reuse dishes simply wipe off the residual oil with 70% ethanol.
6. To reduce anoxia during longer timelapse imaging the Voltalef or Halocarbon oil can be saturated with oxygen. To achieve this, transfer 1 ml of oil into a microcentrifuge tube and gently bubble oxygen into the oil for about 1 min. Keep the tube tightly capped until the oil is used.
7. The laser power will have to be titrated to generate a wound of an appropriate size. If too large, the wound will not heal in the 3–4 h window prior to embryonic hatching.
8. Timelapse imaging of epithelial wound closure should be watched and subtle focal adjustments down into the sample will be required as the epithelium is sealed shut.
9. Larger wounds can be made, which will result in the recruitment of more hemocytes.
10. More complicated analyses, such as kymography, are possible using this model system with simple ImageJ plugins. Furthermore, the dynamics of intracellular components can be measured with the use of other fluorescent probes. These more complex measurements require further imaging optimization and an enhancement of the temporal resolution. We expect that even the most complex of migration analyses that are often reserved for in vitro models, such as Fluorescent Speckle Microscopy (13), will in the future be possible in these *Drosophila* inflammation and wound assays.

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Chapter 10

Neutrophil Motility In Vivo Using Zebrafish

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Summary

Zebrafish have emerged as a powerful model organism to study neutrophil chemotaxis and inflammation in vivo. Studies of neutrophil chemotaxis in animal models have previously been hampered both by the limited number of specimens available for analysis and by the need for invasive procedures to perform intravital microscopy. Due to the transparency and cell permeability of zebrafish embryos these limitations are circumvented, and the zebrafish system is amenable to both live time-lapse imaging of neutrophil chemotaxis and for screening of the effects of chemical compounds on the inflammatory response in vivo. Here, we describe methods to analyze neutrophil-directed migration toward wounds using both fixed embryos by myeloperoxidase activity assay, and live embryos by time-lapse microscopy. Further, methods are described for the evaluation of the effects of chemical compounds on neutrophil motility and the innate immune responses in zebrafish embryos.

Key words: Zebrafish, Neutrophil, Chemotaxis, Myeloperoxidase activity assay, Time-lapse microscopy

1. Introduction

The zebrafish, *Danio rerio*, has become a powerful model organism to study cellular activities and cell migration in vivo (1). Two significant advantages of the zebrafish system are (1) the large numbers of embryos that can be obtained, which facilitates quantitative studies, and (2) the optical transparency of these embryos, which enables microscopic observation without the need for surgery or invasive procedures. Another advantage of zebrafish embryos is the permeability of the embryos to chemical compounds that has enabled high-throughput screening of the effects of small molecules (2–4).

Zebrafish embryos have also recently emerged as a highly effective model system to study hematopoietic cells (5, 6) and the

immune response in vivo (7–12). Zebrafish develop neutrophils, which can be identified by their amoeboid shape and cytoplasmic granules (13), and in fixed samples by the expression of the neutrophil-specific protein myeloperoxidase (MPO, also referred to as myeloid-specific peroxidase, MPX) (14, 15). Neutrophil development beyond 2 days post fertilization (dpf) occurs within the Caudal Hematopoietic Tissue (CHT, *see* Fig. 1e), a recently described region of early hematopoietic development (16).

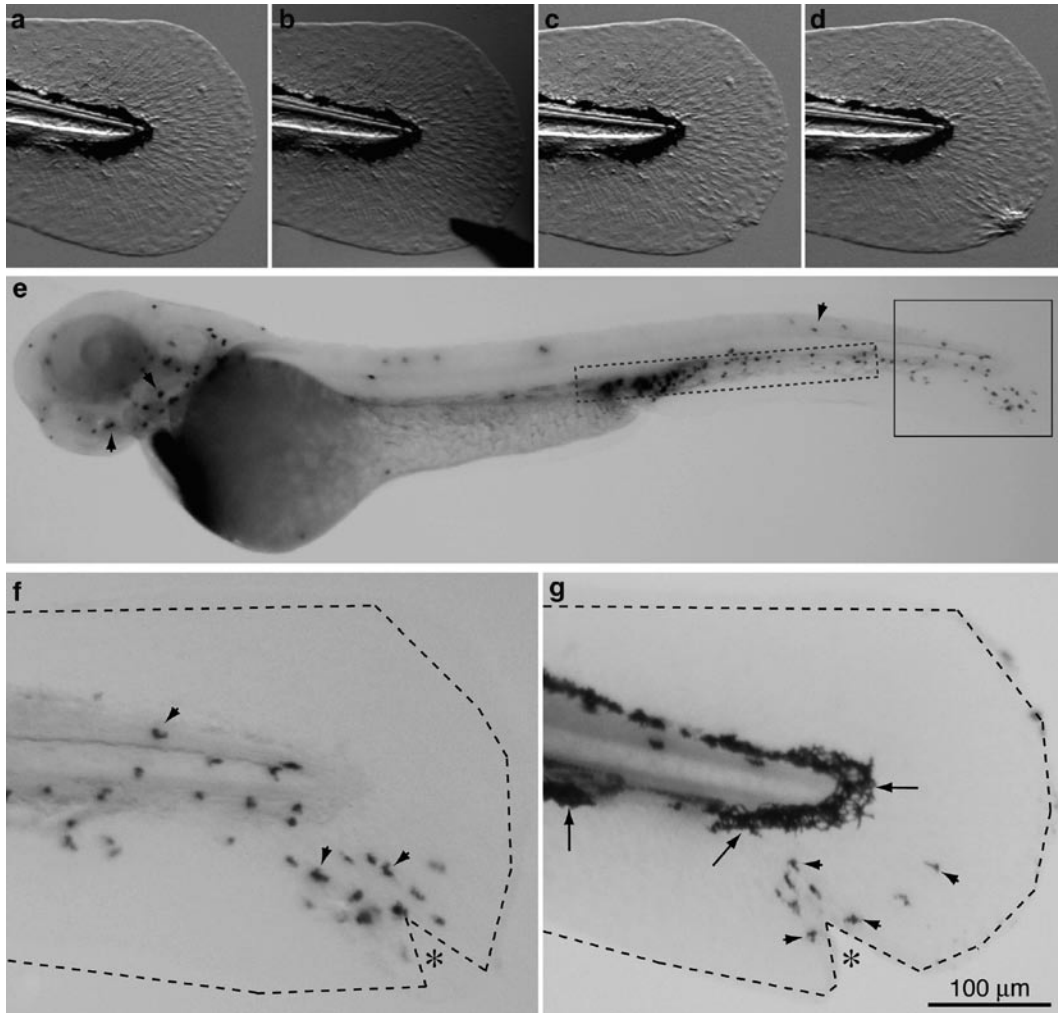


Fig. 1. Zebrafish embryo wounding and MPO activity assay. (a–d) Sequential images of a wound being induced in the distal tailfin of a 3 dpf embryo. (a) Tailfin prior to wounding. (b) The tip of a 25-gauge needle (lower right corner) is pressed down onto the tailfin, against the dish. (c) The tailfin immediately following the wound. (d) The tailfin ~5 min after wounding – note that fin cells immediately around the wound have contracted and/or rounded up. (e–g) Embryos wounded and fixed at 2 h post-wound, then labeled by the MPO activity assay; individual neutrophils are indicated with arrowheads. (e) PTU-treated embryo at 3 dpf, whole body view – at this stage neutrophils are mainly found in the head, around the heart, and in the Caudal Hematopoietic Tissue (dashed box). (f) Higher magnification of boxed region in (e), dashed line outlines tailfin – note the accumulation of MPO-positive neutrophils around the wound (*). (g) 3 dpf embryo tailfin as in (f), only without PTU treatment; note pigment (arrows), which can usually be differentiated from MPO-positive neutrophils.

Previous studies indicate that there are at least two inflammatory leukocytes, neutrophils and macrophages, that are recruited in response to tissue wounding in larval-stage embryos (8, 9). The quantification and detection of neutrophils recruited to tissue wounding can be easily performed using methods to detect endogenous MPO activity using modifications of previously described methods (7, 15). Here, we describe the use of a commercially available kit for the detection of myeloperoxidase activity and quantification of neutrophil recruitment in fixed zebrafish embryos (9, 17). Zebrafish neutrophils can also be detected in fixed embryos by using Sudan Black staining (13, 18) or in situ hybridization of MPO mRNA (14, 15). Further, we will describe methods to analyze neutrophil migration in live zebrafish embryos using time-lapse microscopy (7, 9). This protocol can be performed using conventional light microscopy and differential interference contrast (DIC) to detect leukocytes, both neutrophils and macrophages, as they directionally migrate to the wound. Alternative methods to detect the specific migration of neutrophils in vivo can be performed using transgenic models of zebrafish that express GFP from the neutrophil-specific promoter, MPO (9, 11). We will also discuss methods that can be used to screen for the effects of chemicals on neutrophil migration and inflammation in vivo.

2. Materials

2.1. Zebrafish Maintenance and Mating

1. Adult zebrafish (AB wild-type strain) can be purchased from the Zebrafish Resource Center (Eugene, OR) or from a local pet store. However zebrafish purchased from a pet store are much more likely to have diseases due to improper husbandry.
2. Adult Fish Water: 90 mg/L sodium bicarbonate (Sigma or Aquatic Ecosystems), 50 mg/L Instant Ocean Salt (Aquatic Ecosystems), 10 mg/L calcium sulfate. Due to differences in local water sources, these parameters should be used as a guideline; in particular, the amount of Sodium Bicarbonate may vary and should be added at a concentration that yields a pH of around 7.5.
3. Small nets for handling adult fish.
4. Mating chambers. Mating chambers are generally clear plastic boxes (0.5–1.0 L volume) with a sieve that separates adult fish from embryos after they have been laid to prevent adult fish from eating the embryos. Chambers can be purchased from several sources. We recommend chambers from Aquatic Habitats, which come with dividers that keep individual fish

separate overnight. Alternatively the bottom of a clear plastic box can be covered with marbles, such that embryos can fall through to the bottom where they are inaccessible to adults.

5. Plastic tea strainer(s) to collect embryos.
6. Plastic 10*cm Petri Dishes, non-Tissue Culture. Dishes can be reused several times. In between uses disinfect by treatment in a mild bleach solution overnight, followed by several washes in water (deionized water) and then drying. After hatching, embryos can stick to fresh plastic Petri dishes, which can possibly damage the embryos and cause an inflammatory response thereby complicating the protocols in this chapter. For this reason we recommend maintaining embryos in reused dishes (which do not seem to stick to embryos) or disinfecting and rinsing fresh dishes as detailed earlier prior to their use for these protocols. Dishes used for chemical treatments or fixation (and the subsequent MPO activity assay) should not be reused for live embryos, as residual amounts of chemicals left on plates may damage or kill embryos.
7. Flexible plastic transfer pipettes, 7-ml total volume.
8. E3 medium for embryos: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_2 , 0.33 mM MgSO_4 , $10^{-5}\%$ methylene blue. We usually make a 60 $\times$ stock of the salts and a 0.01% stock of methylene blue, both of which are stored at 4°C. 1 $\times$ E3 is kept at room temperature and can be brought to pH ~7 by addition of 0.05N NaOH or 1 M Tris-HCl, pH 7.6.
9. Dissecting microscope for embryo observation.
10. Incubator set at 28.5°C.
11. *N*-Phenylthiourea (PTU, Sigma; also known as phenylthiocarbamide) can be added to E3 medium at 0.2 mM to prevent pigment formation. A 50 $\times$ stock (10 mM) can be made in distilled water and stored at room temperature. *PTU is toxic*, so wear gloves at all times when handling stock solution or plates containing media. For the benefit of others, be sure to indicate plates that contain PTU (e.g., we place a large green “+” sign on plates containing PTU).
12. Pronase (Sigma). Stock solution of 20 mg/ml can be aliquoted and stored at -20°C or -80°C. Working solutions of 1–4 mg/ml in E3 can be stored as 10–15 ml aliquots at -20°C; warm to ~30°C before using with embryos. Working solutions can be refrozen and reused several times, but with decreasing effectiveness.

2.2. Endogenous MPO Activity Assay

1. Tricaine (Ethyl 3-aminobenzoate, Sigma; also known as MESAB). Stock solution of 4 mg/ml is made by dissolving 400 mg Tricaine and 1 g Na_2HPO_4 into 100 mL deionized water; store at 4°C. Dilute to 0.1 mg/ml in E3 for working solution.
2. Needle, 25 gauge.

3. *Phosphate-Buffered Saline (PBS)*. 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.3. It is useful to prepare a 10× stock of this buffer for dilution of fixative and other reagents.
4. Paraformaldehyde (16%), individual vials (Electron Microscopy Sciences). Dilute to 4% in 1× PBS (from 10× PBS stock) for fixation.
5. Tween-20 (Fisher).
6. Leukocyte Peroxidase Kit (Sigma). Components: Peroxidase Indicator Reagent (store at 4°C), 10× Trizmal, pH 6.3, buffer (store at room temperature).
7. TT buffer: 1× Trizmal of pH 6.3, 0.01% Tween-20.
8. 30% hydrogen peroxide (store at 4°C).
9. Dimethyl sulfoxide (DMSO) (Sigma) (if needed for compound solubility).
10. Bovine serum albumin (BSA) (Sigma) (if needed for compound solubility).
11. Plastic 3-cm or 6-cm Petri dishes, non-Tissue Culture.

2.3. Live Microscopy of Zebrafish Embryos

1. E3 containing 0.1 mg/ml Tricaine.
2. Needle, 25 gauge.
3. 1% low-melt agarose in E3.
4. 3-cm plastic Petri dishes (for upright microscope).
5. 3-cm plastic Petri dishes with glass bottom (for inverted microscope). We make these by drilling a hole about 1.8 cm in diameter in the bottom of the Petri dish. An acid-washed coverslip with a diameter of about 2.2 cm is glued in place over the hole using ultraviolet curing adhesive (Norland Products, Inc.).
6. Pipette tip is used to position embryos in agarose.
7. Microscope equipped with differential interference contrast (DIC) and/or fluorescence imaging capabilities. We use either a Nikon Eclipse TE300 inverted microscope equipped with epifluorescent illumination or a Fluoview FV1000 FV10-ASW confocal laser scanning microscope, both equipped with 20× and 60× objectives.

3. Methods

3.1. Zebrafish Maintenance and Mating

1. Maintain adult zebrafish according to established husbandry protocols (19). Male fish are somewhat slimmer and have yellow underside, while females will have a white, extended belly when they are ready for mating.

2. Set up crosses of adult fish on the afternoon prior to when you want embryos. Fill mating chambers with fish water, and using small nets place one male and one female in each mating chamber; cover with a lid (with holes for air) to prevent fish from jumping out overnight. The number of crosses to be set up is dependent on the number of embryos you want for experiments. General guidelines to keep in mind are that most of the time only 50% of crosses result in embryos, and that clutch sizes range from 50 to 200 embryos. Many commercially available mating chambers also come with dividers to keep fish separate, thereby allowing one to control the time of mating, and small plastic shrubs, which can be added to provide a place for a fish to hide from a more aggressive fish.
3. The next morning, remove dividers (if included) and allow fish to mate. Zebrafish are induced to breed by the morning light turning on; male fish will aggressively swim alongside females, which will release embryos that are then fertilized by the nearby male. Embryos will fall to the bottom of the chamber, where they should be left for 5–10 min to ensure fertilization. If fish do not mate, we have found that embryo production can be helped by combining tanks (resulting in crosses of two males with two females) or by raising the internal sieve to slightly immobilize the female fish.
4. After embryos are laid, remove adult fish to a separate chamber and collect embryos by pouring the water and embryos through a plastic tea strainer. Tap embryos into a 10-cm Petri dish containing E3 (1/2–3/4 full, enough to sufficiently cover embryos), if necessary use a squirt bottle or transfer pipette filled with E3 to remove all embryos from strainer. Alternatively embryos can be moved from mating chamber to Petri dish using a transfer pipette – if this is done be sure to change E3 in dish after all embryos have been transferred. Remove and discard all debris (fish scales, feces, etc.) from dishes with a transfer pipette.
5. For larger clutches use a transfer pipette to divide embryos into additional dishes, such that no more than ~100 embryos are in each dish. Place dishes with embryos in incubator set at 28.5°C; keep embryos at this temperature at all times unless otherwise noted.
6. At around 6 h post fertilization (hpf) or later in the afternoon observe embryos using a dissecting microscope; remove and discard unfertilized embryos, which will have single, misshapen cells that have not divided or gone through development. Unfertilized embryos will eventually lyse and may promote bacterial growth.

7. At 24 hpf, remove and discard unfertilized or dead embryos, which will appear white and translucent. Replace E3 media by tipping dish and allowing embryos to collect at edge of plate, and then remove enough media such that embryos are still completely covered; after this add fresh E3. If embryos lacking pigment are desired, exchange media at this point with E3 containing 0.2 mM PTU. Wear gloves when handling media containing PTU.
8. Embryos will naturally hatch from chorions at 2.5–3 dpf; check periodically for discarded chorions, which should be removed. Replace media as needed, keeping the level at one-half to three-quarter full. If desired, chorions can be removed after 24 hpf by treatment with Pronase (*see* [Note 1](#)). Hatched embryos can be moved among plates using a flexible transfer pipette; transfer embryos gently to avoid injury, especially when expelling embryos from pipette.

3.2. Endogenous MPO Activity Assay Following Wounding

1. Incubate embryos at 28.5°C to 2–4 dpf. At these stages embryos will lie flat on their sides and neutrophil development will have commenced. We prefer to use embryos at 3–4 dpf because the embryo is more “flat” than 2 dpf (due to reduction in the size of the yolk sac) and thereby easier to wound. At 5 dpf, embryos inflate swim bladders and do not rest on their sides, making media exchange and wounding more difficult. For statistically significant results we recommend assaying *at least* 20 embryos per condition tested; higher numbers may be required to address the variability of neutrophil recruitment.
2. Prior to wounding, chemical compounds can be added to the media to pretreat embryos in order to make sure embryos are suffused with the compound; concentration and duration of pretreatment would need to be determined empirically (for notes on compound solubility and long-term treatments, *see* [Note 2](#)). To reduce the amount of compound used, embryos can be transferred to smaller dishes (e.g., 3 cm or 6 cm diameter); if this is done maintain lower numbers of embryos per dish (~5 embryos per ml media). If a compound is to be tested, a vehicle control plate should be set up in parallel using an equivalent number of embryos. Dilute compound (or vehicle) into E3 in a separate container without embryos prior to replacing embryo media as detailed in [Subheading 3.1, step 7](#). *Hereafter “media” will refer to E3 with or without compound.*
3. Anesthetize embryos by placing in media containing 0.1 mg/ml Tricaine for ~5 min at room temperature.

4. Wound the distal tailfins (*see Note 3*) with the tip of a 25-gauge needle (*see Fig. 1a–d*). Attempt to make wounds of uniform size, as different-sized wounds will result in differing degrees of inflammatory response (*see Note 4*). Make note of the time of wounding for accurate fixation time points.
5. Wash embryos 1–2 times in media lacking Tricaine; then place at 28.5°C until fixation.
6. At desired time point (*see Note 5*), fix embryos in 4% paraformaldehyde/PBS for 2 h at room temperature. If embryos are floating, Tween-20 can be added to 0.01% to keep embryos submerged. To reduce the amount of fixative (and subsequent reagents) used, embryos can be transferred to smaller dishes (e.g., 3 cm or 6 cm diameter) or 1.5-ml tubes for the remainder of the protocol.
7. Remove paraformaldehyde and dispose of properly. Wash fixed embryos 3 × 5 min in deionized water (*see Note 6*).
8. Prepare TT buffer; set aside sufficient TT buffer (e.g., 2 ml per 3 cm plate) for diluting substrate in **step 10** of this section.
9. Wash fixed embryos 3 × 5 min in TT buffer; following the third wash place embryos at 37°C. We have found that equilibration in TT buffer – especially the third wash at 37°C – is critical for efficient, optimal labeling.
10. While embryos are incubating at 37°C, dissolve Peroxidase Indicator Reagent in TT buffer at 1.5 mg/ml (*see Note 7*) and warm to 37°C. Substrate/TT solution should eventually take on a purple/brown color.
11. Immediately before reaction add hydrogen peroxide (to 0.015%) to substrate/TT solution and mix.
12. Remove TT from embryos, replace with substrate/TT/hydrogen peroxide mix, and place plates at 37°C. Monitor development intermittently on dissecting microscope – this should be most obvious within the CHT (*see Fig. 1e*) and near wounded tailfins (*see Fig. 1f–g*).
13. When individual MPO-positive neutrophils can be discerned, stop reaction by washing labeled embryos in PBS several times. *Do not allow the reaction to overdevelop*, as the precipitate that is produced may extend beyond cell boundaries and cause individual MPO-positive cells to overlap. This will hinder counting individual neutrophils that are close together, which is especially critical at the wound.
14. Count number of MPO-positive cells at (or in the vicinity of) the wound for each sample (*see Fig. 1f, g*).
15. For long-term storage leave embryos in 4% paraformaldehyde/PBS (or other fixative) at 4°C.

3.3. Live Microscopy of Zebrafish Embryos After Wounding

1. Prepare microscope for image acquisition and set room temperature (or closed system of microscope) at 28–30°C.
2. Place embryos in 3-cm Petri dish, in E3 containing 0.1 mg/ml Tricaine to anesthetize fish. Add chemical compound for pretreatment as in **Subheading 3.2, step 2**. (Hereafter “media” will refer to E3 containing Tricaine, with or without compound.) If the effect of the compound is reversible, it can be washed out simply by replacing the compound-containing media with fresh E3.
3. Heat low-melt agarose in microwave oven, using short bursts, just enough to melt agarose. We use 0.8–1% agarose made up in E3 and Tricaine is added to 0.1 mg/ml after heating. Cool to around room temperature. Movies can also be made with embryos in media alone, but shifts in position are much more likely. Furthermore, certain compounds may not penetrate agarose, which may require keeping embryos in media (*see Note 8*). If embryos are not embedded in agarose, skip to **step 5**.
4. Take media off embryos (not all – do not allow embryos to dry) and replace with agarose – enough to cover. Swirl dish to evenly distribute embryos and quickly orient embryos while agarose is still molten using a pipette tip (*see Fig. 2a*). For imaging of the fin it is important to try to get embryos as flat as possible on the bottom of the dish. If multiple movies are to be taken at once (i.e., with automated stage) you may want to orient embryos parallel, in same position.
5. Allow agarose to harden, fixing embryos in position. Cover top of agarose with media. Embryos will remain perfectly fine in agarose for more than 12 h as long as the agarose is covered with media.
6. Orient dish on microscope stage, bring embryos into focus. If doing fluorescence microscopy using the zMPO:GFP transgenic line or other transgenic line, check GFP level of multiple embryos to select optimal embryo. Set up camera for acquisition of either DIC or fluorescence images, or both if your microscope has dual imaging capabilities. This step is done to set embryo position for quick placement after wounding.
7. Using dissecting microscope, wound ventral tailfin of the embryo using 25-gauge needle (*see Fig. 2b*, and **Notes 3 and 4**). If using an inverted microscope the needle can come in from above (imaging below). For standard microscopy come in at ~45° angle relative to the plate and try not to disturb agarose. If the agarose becomes too distorted it may cause the fin to shift (as agarose repositions) during image acquisition.

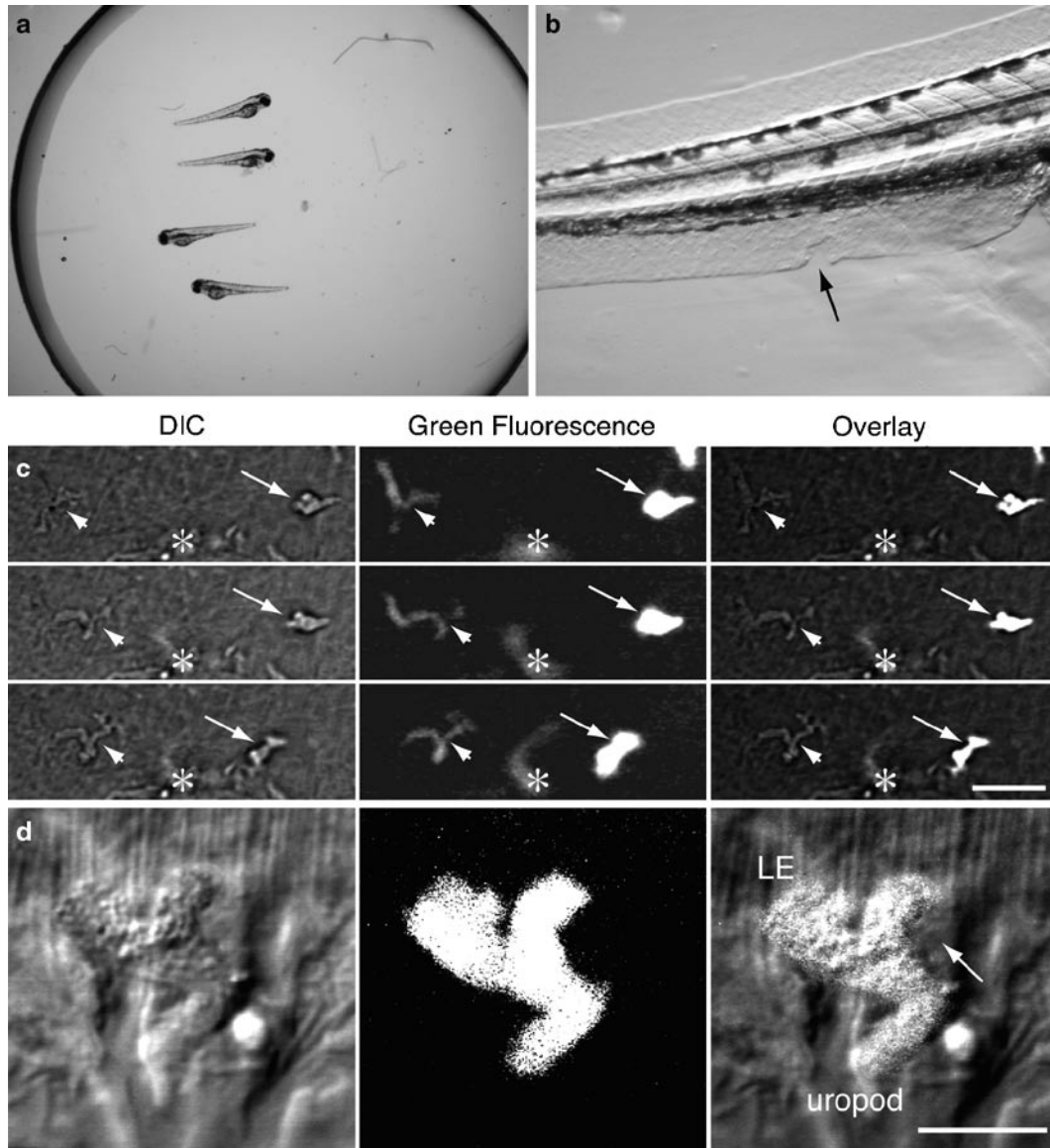


Fig. 2. Time-lapse microscopy of Zebrafish Neutrophils. (a) Four zebrafish embryos embedded in 1% agarose in a 3.5-cm glass-bottomed Petri dish, prior to wounding. (b) A wound (arrow) was made in the ventral fin of an embedded embryo from (a) using a 25-gauge needle (as in Fig. 1b) – note slight disruption of agarose to the right of the wound, where needle was inserted. (c) A sequence from a movie (20 $\times$ objective) taken after wounding of a zMPO:GFP embryo in the ventral fin; each column depicts three sequential time points, with 30 s in between each frame. Shown for each time point are DIC (left frame), corresponding green fluorescence (middle frame), and overlay (right frame). A macrophage (arrowhead) and a neutrophil (arrow) can be distinguished by morphology (left frame) and by GFP expression (middle frame) as they migrate towards the wound (*). Scale bar = 25 μ m. (d) High magnification (60 $\times$ objective with optical zoom) DIC, green fluorescence and overlay images of a polarized neutrophil migrating near a wound (arrow denotes direction of migration) made in the ventral fin of a zMPO:GFP embryo. Granules can be seen streaming near the leading edge (LE) of the cell and GFP expression clearly denotes the boundaries of the neutrophil. Scale bar = 10 μ m.

8. Replace dish on microscope stage as in **Step 6** and begin acquiring DIC and/or fluorescent images as soon after wounding as possible. We normally acquire using a 20× objective at 30 s per frame (*see Fig. 2c*), although shorter timespans can be used for greater resolution of single-cell features such as pseudopod extensions. Movies generally run about 3 h, but can be continued to further observe the inflammatory response (*see Note 9*). If the goal is to observe neutrophil morphology during migration in high resolution (*see Fig. 2d*) or to observe the subcellular localization of a fluorescent protein during migration, a 60× objective can be used, although the distance over which a neutrophil can be tracked will be much more limited. Macrophages and neutrophils can be distinguished by morphology using DIC microscopy (*see Note 10*) or by expression level of GFP if using the zMPO:GFP transgenic line (*see Note 11*).
9. Capture images using software such as MetaMorph or ImageJ, which is freely available from NIH. Minor shifts in embryo position can be corrected by realigning adjacent images using the “Align” tool in MetaMorph, with areas of pigmentation serving as convenient markers.
10. For statistical analysis of leukocyte motility, capture enough movies (minimum of $N = 3$ per treatment) such that 20–30 leukocytes can be accurately tracked for each treatment. We often use the “track point” function of MetaMorph to track cells.
11. Cell-tracking data can be copied to MS Excel to facilitate analysis. Important migration characteristics such as average cell velocity and directionality index can be calculated and compared (*20, 21*). A convenient way to generate a figure for each movie is to use the graph function of MS Excel to generate tracks that can be overlaid onto images acquired from the microscope.

4. Notes

1. To remove embryos before 2.5 dpf, incubate in Pronase (1–4 mg/ml in E3) for ~5 min at room temperature, or until first embryos come out of chorions. Since prolonged treatment with pronase may damage embryos, immediately wash 3× in E3 (~20 ml per wash), during which remaining embryos should come out of chorions, which should be removed from media. Pronase working solution can be reused for additional plates; remove media from embryos and replace with

working solution, each time attempt to limit the number of chorions in the working solution by removing as soon as the first embryos are observed to be out of chorions.

2. If solubility becomes a problem for a particular compound, DMSO can be added (up to 1%) to media. BSA can also be added to media up to 0.2% (or higher, as determined) but should not be used for extended incubations due to potential bacterial growth. Further additions to media for compound screening have also been described (4). For long-term treatments with compounds, one should consider omitting PTU treatment, as this may interact with some compounds and cause complications.
3. For wounding, the most convenient body section to work with is the distal tailfin, which provides a large, flat surface area that facilitates subsequent imaging. Alternatively wounds can be made in the ventral section of the tailfin (*as in Fig. 2b*) just below the caudal hematopoietic tissue (CHT, *see Fig. 1e*), which is slightly more difficult than wounding the distal tailfin but gives leukocytes a shorter distance to travel. For time-lapse microscopy it is often useful to wound in this area, as this potentially enables the visualization of random migration within the CHT in the same movie. If the zMPO:GFP transgenic fish is being used, we recommend wounding below the CHT (*see Fig. 2b*) rather than in the distal tailfin, which contains several nonhematopoietic cells that misexpress the transgene and thereby hinder neutrophil tracking.
4. The size of the wound usually (but not always) corresponds to the degree of leukocyte recruitment, with smaller-sized wounds (*as seen in Fig. 1c, d*) resulting in less recruitment than a moderately sized “wedge” taken out the tailfin (*as seen in Fig. 1f, g*). Transection of the tail has also been performed (11, 12, 15); however, we find that wounds of this size can lead to a large response that can impair tracking of individual cell motility. For quantitative assays using embryos that will be fixed, effort should be made to make consistently sized wounds in the same location of each embryo. To address the variability in the assay, we recommend assaying *at least* 20 embryos per condition tested. Further variations in leukocyte recruitment due to strain background or media sterility (7) should encourage individual researchers to empirically determine the size of wound that yields a reasonably consistent result prior to performing assays of chemical compounds or transient gene knock-down (18). Wound size is an essential factor to consider for time-lapse microscopy, since a large wound may cause an overwhelming leukocyte response that could hinder analysis of individual migrating cells for cell tracking.

5. We and others have previously assayed and discussed the kinetics of the neutrophil response to tailfin wounding (7, 9). It is important for individual researchers to experimentally determine the optimal postwound time to assess the neutrophil response. In general we have found that 2 h post wound (hpw) is a very good starting point to assay leukocyte recruitment, but earlier or later time points may be better depending on the size of wound. It therefore may be useful to perform multiple time points (e.g., 1 hpw, 2 hpw, 3 hpw, etc.) using similar numbers of embryos to get a more accurate assessment of the neutrophil response. If embryos are fixed at multiple time points we recommend concurrently carrying out the fixation and washes (steps 6 and 7 of Subheading 3.2) for each sample, then leaving each sample in the first TT buffer wash (step 9 of Subheading 3.2) until the remaining steps can be performed simultaneously on all samples. Samples can be left in TT buffer overnight at 4°C if necessary, and overnight fixation at 4°C has also been reported (17). We do not recommend leaving fixed embryos in PBS for extended periods, as this seems to result in a reduction of MPO activity.
6. When doing media exchanges during MPO activity assay, take off media down to the level of the embryos while still keeping them submerged; embryos exposed to air may become slightly dried out, which might reduce MPO activity. Beyond ~4 dpf embryos inflate swim bladders, which may make media exchange difficult as embryos do not sink to the bottom of the dish (or tube), and may be accidentally taken up by the pipette. To deal with this we have found that fixing a plastic 10- μ l pipette tip to the end of a transfer pipette aids media exchange by reducing the size of the opening. If this is done take special care to prevent embryos from being sucked into or stuck to the tip, which may damage the fixed embryos.
7. The Sigma Leukocyte Peroxidase kit contains single vials of Peroxidase Indicator Reagent that are aliquoted for single uses. We have adapted this kit for multiple uses of single vials of the Indicator Reagent, which consists of two solid components (one white, one purple) that are not uniformly mixed. We have found that 1.5 mg (per ml TT) of the mixed Indicator Reagent is sufficient for labeling as long as there appears to be adequate amounts of each solid component. Alternatively, an entire vial of Indicator Reagent can be dissolved in 50 ml TT, aliquoted without hydrogen peroxide and stored indefinitely at -80°C for future use.
8. Some drugs may not penetrate agarose well. We have had some good results by placing embryos *on* agarose, in particular

if a small well is made (e.g., by tearing out a small bit of agarose with a pipette tip) to fit the yolk sac and partially hold the embryo in place. Note that while this method would work for upright microscopy, it would not work as well with an inverted microscope. Other groups have made up agarose to contain the drug of interest (22), potentially facilitating drug delivery through the agarose. Alternatively, after embedding the embryo agarose surrounding the head can be carefully peeled away, exposing the embryo directly to the drug containing media while leaving the body and fins encased in agarose. In any case, the dose and method of drug delivery to embryo is best determined empirically.

9. We generally observe the commencement of neutrophil migration toward the wound at 30 min postwounding but often it begins sooner. If neutrophil recruitment does not occur by 60 min postwounding we tend to switch to a different embryo with a fresh wound. The timing of neutrophil recruitment will vary between embryos depending partially on the size and severity of the wound induced (*see* [Notes 4](#) and [5](#)).
10. Several morphological characteristics can be utilized to identify the two types of leukocyte that are recruited to a tailfin wound (9). Neutrophils are usually the first cell type to migrate to the wound, and can be identified by a relatively compact amoeboid cell body that is distinctly granular in appearance; at high magnifications neutrophil granules can be seen throughout the cytoplasm (*see* [Fig. 2d](#)). Neutrophils migrate very efficiently and with a polarized morphology (*see* [Fig. 2c](#) and [d](#)). Usually later in the wound response, a second cell type is seen to migrate toward wounds. Based on immunolabeling and functional observations (Mathias, Dodd, Walters, unpublished observations) we believe that these cells represent a subset of (but not necessarily *all*) macrophages. During migration this macrophage cell type takes on an elongated morphology (*see* [Fig. 2c](#)) and migrates without obvious polarization and much less efficiently than neutrophils. This movement often occurs by the extension of multiple pseudopods followed by flowing of the cell body into a single pseudopod (Mathias, unpublished observations). Macrophages often have ingested dots of pigment that move along with them (often trailed) during migration.
11. We have found that the level of GFP expression in a leukocyte can be used to distinguish neutrophils and macrophages in the zMPO:GFP transgenic line. At 3 dpf or later, the brightest and most obvious cells are neutrophils while macrophages express much lower levels of green fluorescence protein (*see* [Fig. 2c](#)).

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Chapter 11

Chemotaxis in Neutrophil-Like HL-60 Cells

Arthur Millius and Orion D. Weiner

Summary

Asymmetric localization of intracellular proteins and signals directs movement during axon guidance, endothelial cell invasion, and immune cell migration. In these processes, cell movement is guided by external chemical cues in a process known as chemotaxis. In particular, leukocyte migration in the innate immune system has been studied in the human neutrophil-like cell line (HL-60). Here, we describe the maintenance and transfection of HL-60 cells and explain how to analyze their behavior with two standard chemotactic assays. Finally, we demonstrate how to fix and stain the actin cytoskeleton of polarized cells for fluorescent microscopy imaging.

Key words: Migration, Chemotaxis, Neutrophil, HL-60, Actin cytoskeleton, Amara transfection, Micropipette, EZ-TAXIS assay

1. Introduction

Directed cell migration toward chemical cues, or chemotaxis, is critical in eukaryotic cells for immune response, wound healing, axon guidance, and embryogenesis ([1](#)). An especially useful model for eukaryotic chemotaxis is the human neutrophil. Neutrophils seek infectious bacteria to engulf at wound sites as part of the innate immune system. They follow gradients of formylated peptides released by the bacteria ([1](#)). Yet, many open questions remain in neutrophil and eukaryotic cell migration. How do cells interpret shallow gradients or initially establish polarity? How is their cytoskeleton rearranged during a turn, and what limits this rearrangement to one part of the cell and not another? What signaling components and circuitry are required to accomplish these processes?

The human promyelocytic leukemia (HL-60) cell line was developed as a simple model system to study neutrophil cell migration without the need to derive cells from primary tissue (2). The immortal cell line can be sustained for extended periods of time in culture and may be frozen for longer-term storage. This is impossible with bone marrow or peripheral blood derived neutrophils. When needed, neutrophil-like cells can be derived from HL-60 cells through differentiation with DMSO (3). Differentiated HL-60 cells (dHL-60) can then be used in chemotaxis assays and to visualize the cytoskeleton of a polarized cell (4). Amaxa nucleofection may be used in dHL60 cells to knock genes down (5–8) or transfect cells with a fluorescent tag to analyze protein localization (our unpublished results). Cell behavior can be analyzed either in response to a point source of chemoattractant (9–11), or using the EZ-TAXIS system, which allows simultaneous measurement of directionality and speed for six different assay conditions (12, 13).

2. Materials

2.1. HL-60 Cell Culture and Differentiation

1. *Culture medium*. Roswell Park Memorial Institute (RPMI) 1640 plus l-glutamine and 25 mM HEPES (Fisher Scientific) supplemented with antibiotic/antimycotic (Invitrogen) and 15% heat-inactivated fetal bovine serum (FBS, Invitrogen); store at 4°C (see Note 1).
2. Dimethyl sulfoxide (DMSO), endotoxin, and hybridoma tested (Sigma).

2.2. Amaxa Nucleofection of HL-60 cells

1. *Recovery medium*. Monocyte medium (Amaxa) supplemented with 2 mM glutamine (Invitrogen) and 20% FBS.
2. 100 µl of transfection solution containing supplement (Amaxa) mixed with 2 µg DNA per reaction (see Note 2).

2.3. Plating Cells for Microscopy

1. Fibronectin from bovine plasma (Sigma); store lyophilized protein at –20°C.
2. *Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS)*. 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (Invitrogen).
3. Gold Seal cover glass 20 × 40 mm No. 1.5 (Fisher Scientific).
4. Lab-Tek 8 well permanox chamber slide (Nunc).
5. *Chemoattractant*. 10 mM formylated Methionine–Leucine–Phenylalanine (fMLP, Sigma) in DMSO; store at –20°C. Prepare 100 µM working stocks in RMPI and store at 4°C up to 1 month (see Note 3).

2.4. Micropipette Assay

1. Glass capillary with filament (World Precision Instruments) (*see Note 4*).
2. *Alexa594 working stock*. 10 mM Alexa Fluor 594 hydrazide (sodium salt, Invitrogen) in DMSO; store at 4°C and protect from light.
3. *Chemoattractant solution*. 200 nM fMLP, 10 μM Alexa594 in RPMI culture medium; protect from light.

2.5. EZ-TAXIScan Assay

1. RPMI culture medium.
2. *Chemoattractant solution*. 200 nM fMLP in RPMI culture medium.

2.6. Staining the Actin Cytoskeleton

1. *Intracellular buffer (2×)*. 280 mM KCl, 2 mM MgCl₂, 4 mM EGTA, 40 mM Hepes of pH 7.5, 0.4% low endotoxin albumin from human serum (Sigma) (*see Note 5*).
2. *Fixation buffer (2×)*. 640 mM sucrose, 7.4% formaldehyde (Sigma) in 2× intracellular buffer; store at 4°C (*see Note 6*).
3. *Stain buffer*. 0.2% triton, 2 μl/ml rhodamine phalloidin (Invitrogen) in 1× intracellular buffer (*see Note 7*).

3. Methods**3.1. Maintenance of HL-60 Cell Culture Line**

1. Unless imaging, all cell work is performed under a biological safety cabinet.
2. HL-60 cells are passaged when the cells reach a density between 1 and 2 million cells/ml in 25-cm² cell culture flasks with 0.2-μm vent cap. Split cells to 0.15 million cells/ml in a total volume of 10 ml prewarmed culture medium. Cells will need to be passaged again after 2–3 days (**Fig. 1**). Maintain cells at 37°C and 5% CO₂ in standard tissue culture incubator (*see Note 8*).
3. Differentiate cells in culture medium containing 1.3% DMSO. Because DMSO is more viscous and denser than culture medium, premix medium with DMSO before adding cells. Cells stop proliferating upon differentiation and typically achieve a density of 1–2 million cells/ml at 7 days postdifferentiation (**Fig. 1**). Cells are most active 5–6 days postdifferentiation, but can still respond even after 8 days (*see Note 9*).
4. To freeze cells, pellet cells by spinning at 100 × *g* for 10 min. Aspirate medium and resuspend in chilled culture medium plus 10% DMSO at 10 million cells/ml. Aliquot 1.8 ml each into cryovials, place in Nalgene cryofreezing container with isopropanol at –80°C for 2 days, and then transfer to liquid nitrogen storage (*see Note 10*).

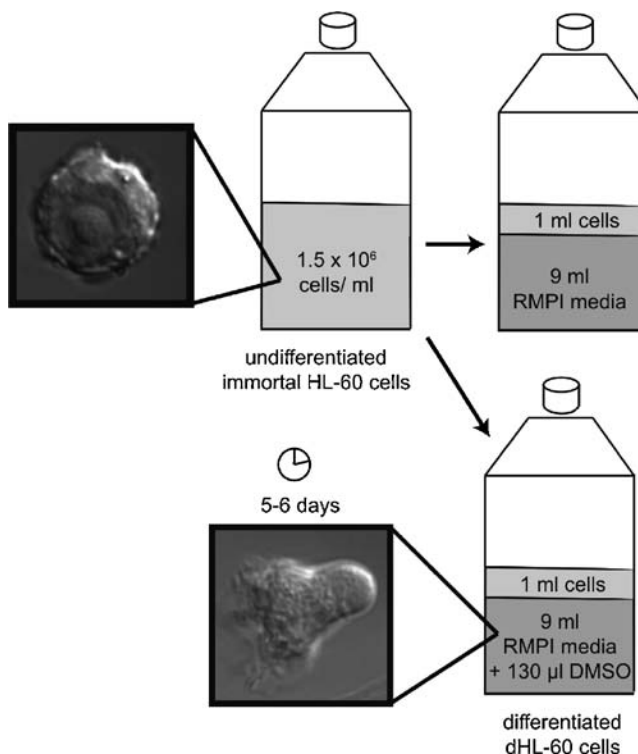


Fig. 1. Passaging and differentiating HL-60 cells. When cells reach a density between 1 and 2 million cells/ml, split to 0.15 million cells/ml in a total volume of 10 ml prewarmed culture medium. Differentiate cells in culture medium plus 1.3% DMSO; cells take ~5 days to become migratory.

5. Thaw cells by quickly warming a cryovial at 37°C just until last bit of ice has melted. Dilute thawed cells in 10 ml of prewarmed culture medium and spin at $100 \times g$ for 10 min. Remove supernatant, resuspend pellet in 20 ml culture medium, and recover in a 75-cm² culture flask.

3.2. Transient Transfection of DNA into HL-60 Cells

1. Prepare ~2 ml of recovery medium per transfection in a 6-well plate and let equilibrate at 5% CO₂ and 37°C for 15 min or more. Add 500 µl of equilibrated recovery medium to an Eppendorf tube per transfection (*see* [Note 11](#)).
2. Spin 5 million cells in a 10-ml Falcon tube at $100 \times g$ for 10 min. Use a separate Falcon tube for each transfection.
3. After spin, remove all medium with aspirator and gently resuspend cells in 100 µl transfection solution with an L-1000 pipette (*see* [Note 12](#)).
4. As quickly as possible, transfer transfection solution to nucleofection cuvette. Electroporate in single-chamber nucleofector on program Y-001.

5. Immediately remove cuvette, use a plastic pipette to obtain 500 μ l recovery medium from Eppendorf tube, flush chamber, and replace medium containing cells in Eppendorf tube.
6. Incubate for 30 min in Eppendorf tube at 37°C (*see Note 13*).
7. Transfer 500 μ l recovery medium and cells with L-1000 pipette to 1.5 ml recovery medium in a 6-well plate. Expression in viable cells occurs in ~2 h with best behavior <8 h after transfection (**Fig. 2**).

3.3. Preparing for live Cell Microscopy

1. Dissolve 1 ml of sterile water in 1 mg fibronectin and let sit at room temperature for 1 h. Avoid shaking or physically mixing because this may denature the fibronectin.
2. Add 4 ml of $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS to fibronectin solution to obtain 200 $\mu\text{g}/\text{ml}$ solution (**Fig. 3a**); store fibronectin solution at 4°C for up to 2 weeks.
3. Remove exterior gaskets from permanox chamber slide and the interior gasket with a razor blade. Discard 8-well plastic walls to expose underlying epoxy mold. With razor, cut single-well epoxy chambers and adhere to glass coverslips (*see Note 14*).

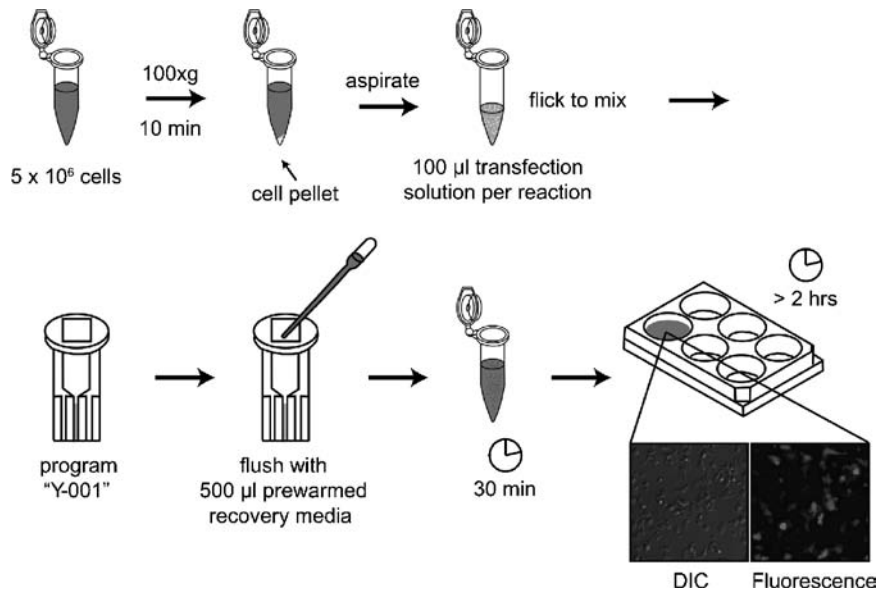


Fig. 2. Transient transfection of HL-60 cells with amaxa nucleofection. Spin ~5 million cells at $100 \times g$. Aspirate supernatant and resuspend pellet in 100 μ l transfection solution per reaction and nucleofect with amaxa program "Y-001." Flush with prewarmed recovery medium and incubate in an Eppendorf tube for 30 min. Transfer to a 6-well dish with 1.5 ml of recovery medium; expression occurs after 2 h. Shown is an example of HL-60 cells 5 h after transfection with GFP visualized with DIC and fluorescence microscopy.

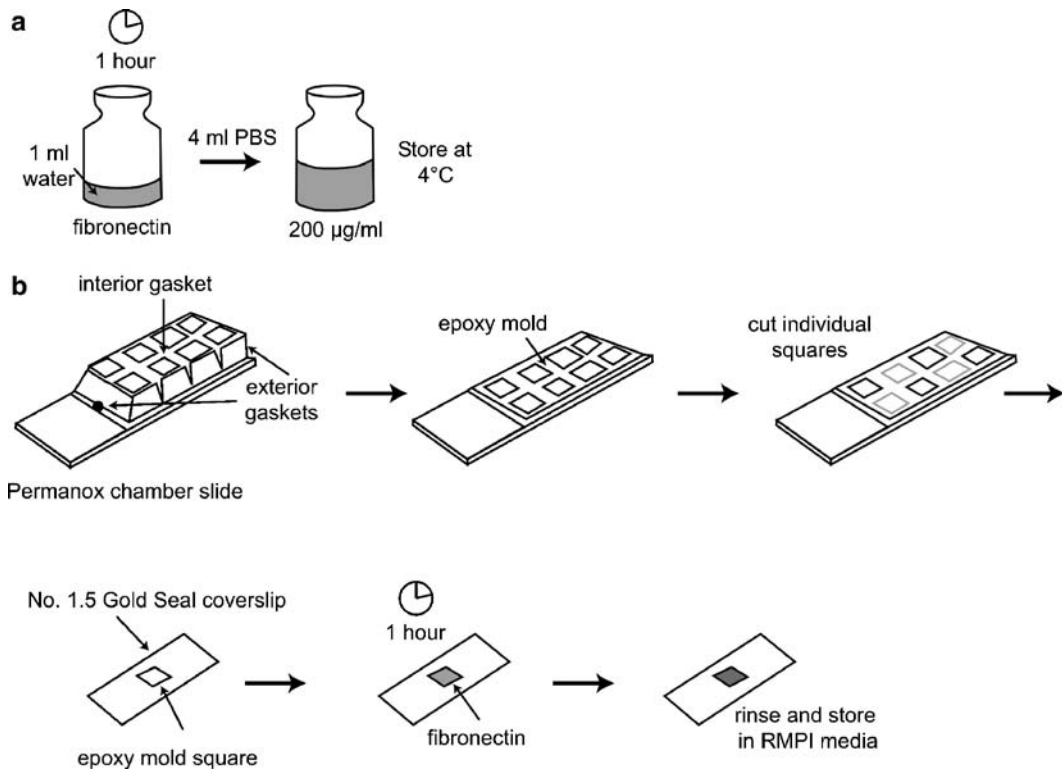


Fig. 3. Preparing a coverslip for live cell microscopy. **(a)** Dissolve 1 mg of bovine fibronectin in sterile water. After 1 h, add 4 ml of PBS and store 200 µg/ml fibronectin solution at 4°C. **(b)** Remove gaskets from plastic permanox 8-well chamber. Cut epoxy mold squares and stick to No. 1.5 gold seal cover glass. Add 125 µl of fibronectin, let sit for 1 h, rinse once with RPMI culture medium, and store in RPMI medium until ready to image.

4. Add 125 µl fibronectin solution to epoxy chamber on coverslip. Incubate at room temperature for 1 h.
5. Aspirate fibronectin solution and rinse once in RPMI culture medium. Add 250 µl RPMI culture medium until ready to plate cells. Fibronectin-coated slides may be stored up to 2 days at 4°C (**Fig. 3b**).
6. For cell plating, remove culture medium on slide and replace with 250 µl differentiated cells (in RPMI culture medium) or transfected cells (in monocyte medium). Incubate at 37°C for 5–15 min (*see Note 15*).
7. Aspirate cells at the corner of the epoxy chamber (avoid touching the coverslip), rinse in RPMI, and replace with 125 µl RPMI. Cells are most migratory at 37°C, but also function at room temperature.
8. During image acquisition, 125 µl of 200 nM fMLP may be added to coverslip (final concentration is 100 nM) to show random migration in response to uniform chemoattractant.

3.4. Micropipette-Stimulated Cell Migration

1. Pull glass filaments on Sutter Model P-87 (Program: heat = 750, pull = 0, velocity = 20, time = 250, pressure = 100, loops 2 or 3 times) to achieve $\sim 2\text{--}3\ \mu\text{m}$ needle diameter.
2. Backfill needles with chemoattractant solution and connect to a needle holder. Flick to remove air bubbles at the tip. The needle holder is held by a micromanipulator and agonist flow controlled by adjusting balance pressure between 0 and 3 psi on a Narishige IM-300 injection system (*see* [Note 16](#)).
3. Place cells seeded on a coverslip on microscope and find focus of cells in brightfield.
4. Orient needle in light path and switch to the back focal plane of the objective (usually labeled “B” for Bertrand lens). Find the needle and lower it until just above the cells; then switch back to the normal focal plane for viewing.
5. Image dye in fluorescence or Total Internal Reflection Fluorescence microscopy using appropriate filter sets with $\sim 20\ \text{ms}$ exposures and near-maximal multiplication on an EM-CCD camera (*see* [Note 17](#)). An example of a cell migrating toward a micropipette filled with chemoattractant is shown in [Fig. 4](#).

3.5. Ascertaining Directionality and Velocity of Migrating Cells with the “EZ-TAXIS” Assay System

1. Place “40 Glass” (41 Glass is used in conjunction with a #1.5 coverslip) in holder base. Fill with 3 ml of culture medium, place small “o” ring beneath wafer housing, and add wafer housing into holder base. Place large “o” ring on top of wafer housing. Gently close the inner lever.
2. Place EZ-TAXIScan chip gently into wafer housing with forceps. Chip should fit snugly on top of glass and between wafer holder (*see* [Note 18](#)). Place rubber gasket (to protect chip) beneath the wafer clamp and place wafer clamp on wafer housing. Gently close outer lever.
3. Place assembled device on top of preheated EZ-TAXIS microscope ([Fig. 5a,b](#)).

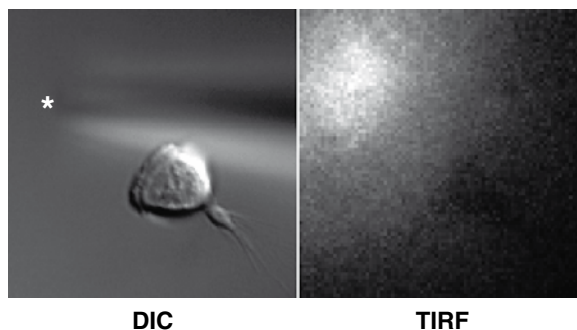


Fig. 4. An example of an HL-60 cell crawling toward a micropipette visualized with DIC and TIRF microscopy. Asterisk indicates micropipette tip.

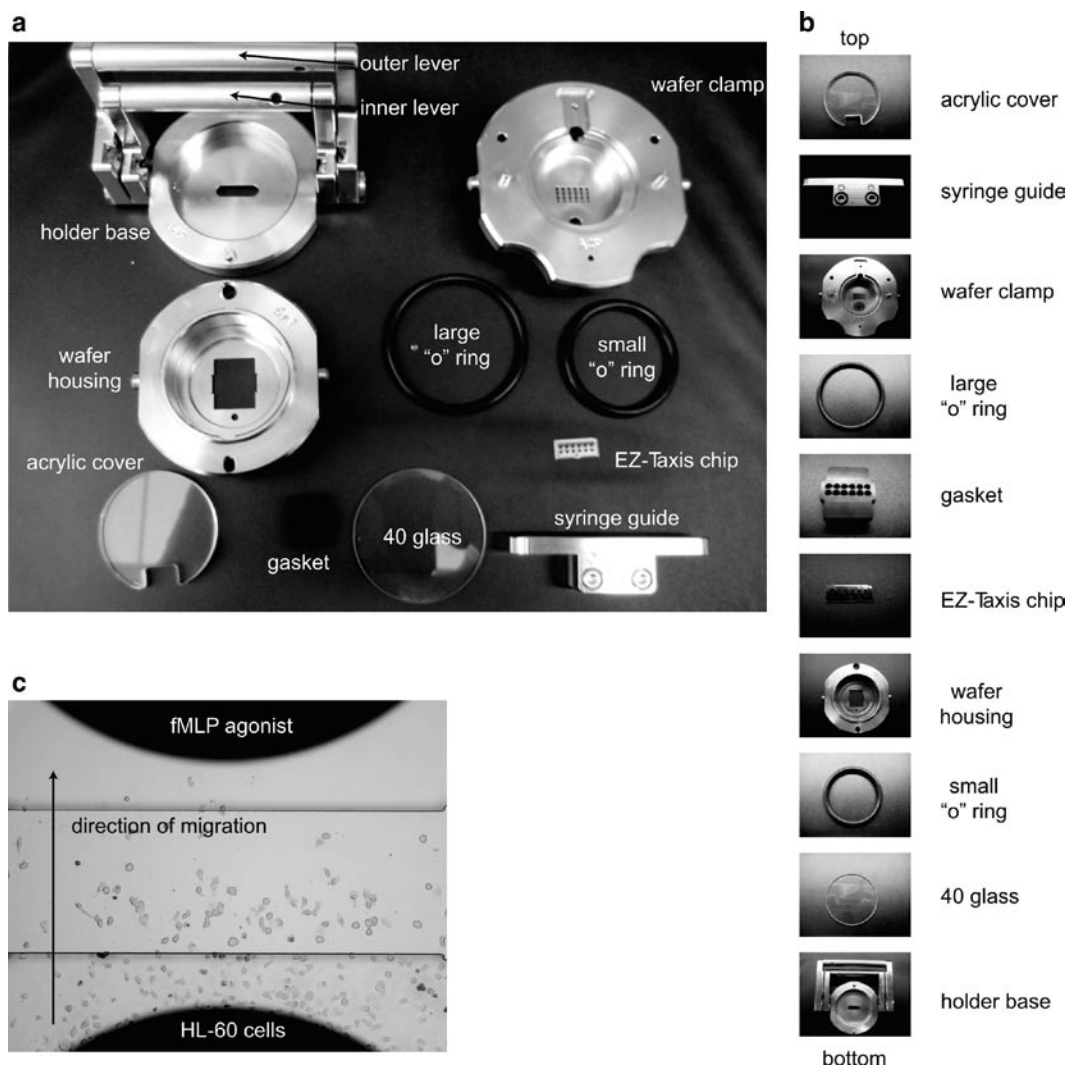


Fig. 5. The components of the EZ-TAXIS system are shown in (a) with the individual components in their order of assembly from *top* to *bottom* shown in (b). (c) An example of HL-60 cells migrating toward chemoattractant in the EZ-TAXIS assay visualized with brightfield microscopy.

4. Using syringe guide, add 1 μ l of cells to lower chamber using microsyringe. Use a plastic pipette to draw cells into imaging surface.
5. Add 1 μ l of 100 nM–1 μ M chemoattractant to upper chamber. Begin image acquisition immediately (Fig. 5c).

3.6. Staining the actin Cytoskeletons

1. Stimulate adherent cells with micropipette or uniform chemoattractant. Alternatively, you can stimulate cells in suspension.
2. Add one volume of 2 $\times$ fixation buffer to cells to give a final concentration of 320 mM sucrose and 3.7% formaldehyde. Fix for 20 min at 4°C (see Note 19).

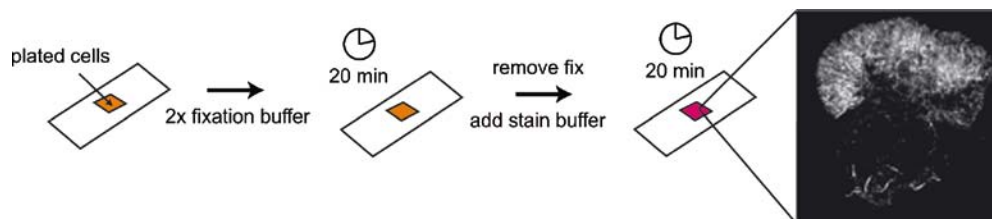


Fig. 6. Staining the actin cytoskeleton. Add 2× fixation buffer to plated cells and fix for 20 min at 4°C. Remove fixation buffer and replace with stain buffer for 20 min; protect from light. Shown is an example of an HL-60 cell stained with rhodamine phalloidin visualized with structured illumination microscopy.

3. Aspirate supernatant (adherent cells) or spin down cells at $400 \times g$ for 1 min and then aspirate supernatant (suspended cells).
4. Permeabilize cells with 125 μ l stain buffer and protect from light for 20 min (**Fig. 6**).
5. Remove supernatant, replace with intracellular buffer, and image (*see Note 20*).

4. Notes

1. HL-60 cells acidify their medium quite rapidly, so HEPES is essential to counterbalance the pH.
2. Poor expression occurs in retroviral LTR-containing vectors.
3. If an exact concentration of fMLP is required, make stocks fresh weekly because formyl peptide will slowly oxidize in aqueous solutions over time.
4. Capillaries with filaments are easier to load than capillaries without filaments.
5. HL-60 cells may be spuriously stimulated with albumin containing endotoxins. It is important to add the albumin fresh to the intracellular buffer and let sit at room temperature for 30 min before mixing because the albumin will oxidize in aqueous solution to products that may also activate HL-60 cells.
6. Sucrose is important to maintain osmolarity.
7. Unlike Alexa Fluor 594, rhodamine phalloidin increases its fluorescence upon binding to actin filaments. This makes it preferable to Alexa Fluor 594. However, for imaging in the green channel, we would use Alexa Fluor 488 or similar fluorophore over the rapidly bleaching FITC.
8. Keep a basin of water on the bottom shelf of the incubator to maintain humidity.

9. Cells may also be differentiated with retinoic acid (14).
10. As an alternative to nalgene freezing chambers, two styro-foam 15-ml Falcon tube holders may be sandwiched around tubes to provide insulation during slow freezing process.
11. Our protocol differs significantly from the standard Kit V protocol from amaxa, because we have optimized for cells to retain chemotactic function (not necessarily highest transfection efficiency).
12. It is important to remove every drop of medium from the Falcon tube because FBS will interfere with transfection. Large-tip pipettes minimize shearing of cells and increases the number of healthy cells post-transfection.
13. Cells will adhere to each other and form a thin, white film during the 30-min incubation in Eppendorf tube. This step increases recovery efficiency. Additionally, cells are fragile after transfection and they behavior poorly if centrifuged.
14. An 8-well chamber will yield four molds. Only bottom surface (side touching permanox chamber slide) of epoxy mold is sticky. Additionally, Lab-Tek 8 well cover glass #1.5 chamber slide (Nunc) may be used for epifluorescence and brightfield microscopy. However, the epoxy that hold the chamber walls to the coverslip is autofluorescent and thus less suitable for TIRF.
15. Cells may be plated in culture medium containing chemoattractant for a higher proportion of adherent cells.
16. We use a universal needle holder (MINJ4, Tritech) connected to a micromanipulator (MM-89, Narishige). Additionally, it is difficult to handle capillaries with gloves on. Once the solution is loaded into the needle, flick the needle vigorously to remove microscopic bubbles. Do not try to force air bubbles through the tip by increasing air pressure. Invariably, the bubbles will become stuck and the needle rendered useless.
17. For quantification of gradient, TIRF imaging is superior to epifluorescence microscopy because TIRF reveals the agonist concentrations in the same plane as the cell. The signal obtained from epifluorescence is a combination of the agonist the cell experiences plus out of focus blur from fluorescent dye above the cell. Confocal imaging can also be used to quantitate the gradient.
18. Chips come in four different sizes (4 μm , 5 μm , 6 μm , and 8 μm) depending on the gap space between the chip and glass. We have used the 4–6 μm chips with success.
19. Formaldehyde can partially permeabilize cells, allowing water influx due to high internal protein concentration.

20. Can repeat wash if background too high. If pressed for time, can immediately image after adding stain buffer. However, this will give high background staining. You can also mount cells in Vectashield (Vector Laboratories) for decreased spherical aberrations and photobleaching.

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Chapter 12

Chemokine Receptor Dimerization and Chemotaxis

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Summary

A broad array of biological responses ranging from cell polarization, movement, immune and inflammatory responses, as well as prevention of HIV-1 infection, are triggered by the chemokines, a family of structurally related chemoattractant proteins that bind to specific seven-transmembrane receptors linked to G proteins. Although it was initially believed that chemokine receptors act as monomeric entities, it has now been shown that they function as oligomers. Chemokine receptor homo- and heterodimers are found on the cell membrane; binding to their ligands stabilizes specific receptor conformations and activates distinct signaling cascades. Thorough analysis of the conformations adopted by the receptors at the membrane is therefore a prerequisite for understanding the function of these inflammatory mediators.

For study of the chemokine receptor conformations at the cell surface, we focus here on conventional biochemical and genetic methods, as well as on new imaging techniques such as those based on resonance energy transfer; we also evaluate in vitro and in vivo methods to determine certain chemokine receptor functions.

Key words: Chemokine, Chemokine receptor, GPCR, Dimerization, BRET, FRET, Chemotaxis

1. Introduction

The chemokines were originally described as specific mediators of leukocyte directional movement. In the current view, however, they are implicated in the movement of several cell types, and participate in functions such as lymphocyte trafficking (1), regulation of T cell differentiation (2), HIV-1 infection (3), angiogenesis (4), development (5, 6), and tumor metastasis (7).

Structural criteria originally led to the classification of the nearly 50 known chemokines in four families, C, CC, CXC, and CX3C. Today scientists refer to them as constitutive chemokines, which are usually regulated during development, and as inducible chemokines, whose expression is regulated mainly during inflammatory processes.

Based on their broad range of functions, the chemokines are easily deduced to be important players in diseases characterized by inflammation and cell infiltration, such as asthma, atherosclerosis, rheumatoid arthritis, multiple sclerosis, colitis, Crohn's disease, experimental autoimmune encephalomyelitis (EAE), and psoriasis, among others (8). Finally, CXCR4 and CCR5 are the two main coreceptors for HIV-1 infection (9); some chemokine receptors also participate in tumor metastasis (7) and transplant rejection (10).

Chemokines exert their effects by interacting with receptors in the membrane of the target cell, the seven transmembrane-spanning G-protein-coupled receptor (GPCR) (11). As is the case for the chemokines, their receptors can also be grouped into two major families, CCR and CXCR, which interact with the CC and CXC chemokines, respectively (12).

Most chemokine signaling experiments were limited to reproducing the signaling cascades associated with other GPCR (13–15). It was initially assumed that chemokine receptor function is governed entirely by G_i -mediated processes (12), since most chemokine functions are blocked in cells pretreated with pertussis toxin (PTx) (16, 17). The chemokine-mediated signaling cascade is nonetheless more complex than originally thought; several studies, including those by our group, demonstrated the existence of chemokine receptor homo- and heterodimers and the functional relevance of these conformations (18–22). Although the difficulties in detecting these complexes using immunoprecipitation methods suggest instability in the absence of ligand, resonance energy techniques clearly show that chemokine receptor dimers form spontaneously in the absence of ligand (21, 23, 24). Several questions remain to be answered, including the dynamic nature of these receptor complexes and the role of distinct ligands in promoting the conformational changes that trigger function. This is particularly important in the case of chemokines, since there is a relative lack of selectivity in ligand binding, with many receptors showing high affinity for more than one chemokine (25), and simultaneous expression of several receptors on the same cell.

Until recently, most assays used to demonstrate receptor oligomerization were based on biochemical approaches such as immunoprecipitation or crosslinking. Alternatively, dominant negative receptor mutants were employed to abrogate wild-type receptor functions by forming nonfunctional complexes (26). New approaches based on energy transfer between fluorochromes

followed by confocal microscopy are now showing us that these biological responses are highly dynamic and extremely dependent on the context in which they take place. These methods exploit important technological advances such as laser light sources, fluorescent probes, and advances in computer science that allow digital imaging and image analysis.

Here we describe conventional biochemical and genetic methods, as well as techniques based on resonance energy transfer to evaluate chemokine receptor conformations at the cell surface. Furthermore, we evaluate *in vitro* and *in vivo* methods to determine chemokine receptor function.

2. Materials

2.1. Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE)

1. *Separating buffer* (1×). 1.5 M Tris-HCl of pH 8.8, 0.1% SDS. Store at room temperature (RT).
2. *Stacking buffer* (1×). 0.5 M Tris-HCl of pH 6.8, 0.4% SDS. Store at RT.
3. Acrylamide/bis solution (29:1) (this is a neurotoxin when unpolymerized; extreme care should be taken to avoid exposure).
4. *N,N,N,N'*-tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA)
5. *Ammonium persulfate*. Prepare a 1.5% solution in H₂O and freeze immediately in single-use (1 ml) aliquots at -20°C.
6. *Running buffer* (10×). 0.25 M Tris Base, 1.92 M glycine, 1% (w/v) SDS (pH should be ~8.5 without adjustment). Store at RT.
7. *Sample buffer* (2×). 200 mM Tris-HCl of pH 6.8, 40% glycerol, 8% SDS, 0.008% bromophenol blue, 6.16% dithiothreitol.
8. *Prestained molecular weight markers*. Full-range Rainbow (Amersham, Buckinghamshire, UK). Aliquot and store at -20°C.
9. *Stripping buffer*. 6 mM Tris-HCl of pH 6.8, 2% SDS, 0.5% β-mercaptoethanol.

2.2. Buffers for Lysis and Immunoprecipitation. Crosslinking Agents (See Note 3)

1. *Lysis buffer*. 10 mM triethanolamine-HCl of pH 8, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 2% digitonin. Store at -20°C and add the 2% digitonin solution just before use.
2. *Washing buffer*. 50 mM Tris-HCl, pH 7.6. Store at RT.
3. Secondary antibodies coupled to agarose beads. Store at 4°C.
4. *Transfer buffer*. 2.5 mM Tris, 19 mM glycine, 20% methanol, 0.037% SDS.

5. *Blocking buffer*. PBS containing 5% nonfat dry milk, or 3% BSA.
6. *Bifunctional agents*. Disuccinimidyl suberate (DSS).

2.3. Expression Vectors for Fluorescent Proteins

Several plasmids with which to fuse the chemokine receptor to fluorescent proteins are commercially available. Some allow inclusion of the fluorochrome at the N-terminal end of the receptor and others at the C-terminal end (e.g., pEYFP-C1, pEYFP-N1 or pDsRed-Monomer N1 vectors from Clontech, Mountain View, CA).

2.4. Fluorescent Labeling of Antibodies: Several Cyanine Dyes are Commercially Available

1. Dimethylsulfoxide (DMSO).
2. *Dissolving buffer*. 100 mM NaCl, 50 mM NaH₂PO₄, 1 mM EDTA, pH adjusted to 7.5 with NaOH.
3. 100 mM NaH₂PO₄.
4. PD-10 desalting columns.

2.5. Colocalization

1. Poly-L-lysine (20 µg/ml).
2. 4% Paraformaldehyde.
3. *Cell-blocking buffer*. PBS supplemented with 1% BSA, 0.1% goat serum, 50 mM NaCl, and 0.05% Tween-20.

2.6. BRET Measurements

1. Coelenterazine H (Nanolight Technology).
2. Multidetector plate reader.

2.7. FRET Measurements

1. Poly-L-lysine.
2. 4% formaldehyde.
3. Microscope with appropriate lasers. Several microscopes are available (Olympus, Nikon, Zeiss, Leica).
4. ImageJ 1.40 g software (NIH).
5. When FLIM determinations are required, use a confocal microscope with a High-Speed Lifetime Module and pulsed laser.

2.8. Migration in Transwells

1. Transwells are available from several commercial sources.
2. Serum-free medium supplemented with 0.1% BSA and 10 mM Hepes.
3. Cell incubator.
4. Flow cytometer.

2.9. Adherent Cell Migration

1. Cell incubator.
2. Migration chambers from Neuro Probe Inc.
3. Collagen IV or fibronectin solution.
4. Crystal violet solution.
5. Serum-free medium supplemented with 0.1% BSA and 10 mM Hepes.

6. *Trypsin/EDTA*. 50 mg/l porcine trypsin, 20 mg/l EDTA (tetra-sodium salt) in Hank's balanced salt solution.
7. Carboxyfluorescein succinimidyl ester (CFSE) solution.
8. 4% formaldehyde.

2.10. Murine In Vivo Cell Migration

1. Calcein-AM, Cell Tracker Green or Cell Tracker Orange.
2. Ketamine/xylazine for anesthesia.
3. Suture or skin staples.
4. 0.85% ammonium hydroxide.

3. Methods

The methods described here allow identification of the GPCR conformation adopted at the cell surface. As any single method alone has intrinsic limitations, most of these should be used in concert with others to obtain meaningful results. Here we outline the most relevant methods, and those that are not commonly used for GPCR studies. The main criticisms of the GPCR oligomerization model nonetheless arise from discrepancies among the results, which are highly dependent on the experimental conditions.

Before attempting analysis of chemokine receptor dimerization, the cell system should be characterized in detail (*see Note 1*). This includes routine testing such as analysis of cell cycle status, cell surface receptor expression and determination of receptor number and affinity constants, especially when cells are transfected with mutant or fluorescently labeled receptors.

3.1. Western Blot and Immunoprecipitation

Although lysis buffer compositions vary (*see Note 2*), the general protocol used for these assays is similar. Cell number should be adjusted, depending on the amount of receptor and/or signaling molecule to be analyzed.

1. Cells (1×10^7 cells/ml), unstimulated or stimulated with the appropriate chemokine, are diluted immediately with 1 ml cold PBS (to terminate stimulation).
2. Centrifuge ($800 \times g$, 5 min, 4°C), wash with cold PBS, resuspend in 200 μl lysis buffer, and incubate 30 min at 4°C with continuous rocking.
3. Precleaning step. Centrifuge ($24,000 \times g$, 15 min, 4°C) and discard the pellet. Incubate the supernatant containing solubilized proteins with anti-immunoglobulin (immunoglobulins of the same species as the immunoprecipitating antibody) coupled to agarose (30 μl /sample, 30 min, 4°C).
4. Centrifuge ($20,000 \times g$, 1 min, 4°C).

5. Incubate the supernatant (90 min, 4°C) with the immunoprecipitating antibody (Ab) (see [Note 3](#)), followed by anti-Ig coupled to agarose (without washing), and incubate (60 min, 4°C).
6. Wash extensively (at least 5 times) with 200 µl washing buffer (50 mM Tris-HCl, pH 7.6).
7. Resuspend the pellet in 50 µl of 2× sample buffer.
8. Procedures for SDS-PAGE, transfer to nitrocellulose membranes and western blot are described elsewhere ([26](#)), and require no major modifications for chemokine receptor analysis (see [Note 4](#)).
9. To reprobe a western blot with another antibody, incubate the nitrocellulose membrane with stripping buffer (90 min, 60°C) (see [Note 5](#)).
10. Wash the nitrocellulose membrane extensively with distilled water.
11. Start the new Western blot analysis by blocking the membrane with an appropriate blocking agent.

3.2. Crosslinking Procedures

1. Stimulate the cells as in [Subheading 3.1](#) and wash by centrifugation ($200 \times g$, 10 min).
2. Resuspend the pellet in 1 ml cold PBS and add 10 µl 100 mM DSS. Prepare the DSS reagent just before use.
3. Incubate (10 min, 4°C) with continuous rocking.
4. Terminate the reaction by adding 1 ml cold PBS and centrifuging ($200 \times g$, 10 min).
5. Lysis step. Wash 3 times with cold PBS and resuspend the pellet in lysis buffer. Continue with immunoprecipitation and western blot analysis as described in [Subheading 3.1](#) (see [Note 6](#)).

3.3. Tagged Receptors

Some chemokine receptors are difficult to detect due to a lack of appropriate tools. In these cases, a peptide can be used for which a monoclonal antibody (mAb) is available; fused to a receptor, the peptide allows detection of the protein of interest. Both ligand-binding affinity and receptor distribution should first be evaluated to assure that the behavior of the tagged receptor is similar to that of the wild-type receptor (see [Note 7](#)). Although several expression vectors are commercially available, homemade epitopes can also be designed and included into the receptor cDNA sequence using standard PCR techniques. For tagged receptors, eliminate the receptor initiation codon and ligate the oligonucleotides that code for the tags selected.

Transfect cells and determine the functional integrity of the individual fusion receptor(s) by conventional flow cytometry and calcium flux techniques. For chemokine receptors, we generally

observe maximum receptor expression at the cell membrane at 48 h after transient transfection (*see* [Note 7](#)).

3.4. Construction of Fluorescently Labeled Receptors

For rapid, easy visualization, one can use fluorescent protein-based constructs and microscopy techniques. Such constructs are typically employed in colocalization analysis and energy-transfer techniques. Receptors are cloned using standard molecular biology techniques into commercially available vectors bearing fluorescent proteins. Insertion of the fluorescent probe in the C-terminal region of the receptor involves elimination of the receptor stop codon, whereas insertion in the N-terminal region requires elimination of the fluorescent protein stop codon. Transfected cells should be analyzed for receptor expression and function (*see* [Note 8](#)).

3.5. Fluorescence Labeling of Antibodies

The cyanine (Cy) dyes are efficient tags for fluorescence labeling, due to their intense fluorescence and low hydrophobicity.

1. Dissolve the contents of a commercial vial ("to label 1 mg of protein") of Cy2, Cy3, or Cy5 in 50 μ l DMSO.
2. Resuspend monoclonal antibody at 1 mg/ml in dissolving buffer.
3. Mix by gently vortexing 10 μ l dye/DMSO and 200 μ l antibody solution.
4. Incubate in the dark (30 min, RT).
5. Add 300 μ l 100 mM NaH_2PO_4 to separate unbound dye. Incubate (30 min, RT), and load onto a PD-10 desalting column pre-equilibrated with dissolving buffer.
6. Wash the column twice with dissolving buffer and elute the labeled protein with 2 ml distilled H_2O (*see* [Note 9](#)).

3.6. Colocalization Assays

These assays are based on detection of light emitted by two different fluorophores, and are evaluated from digital images that show the presence/absence of the same pixel in two distinct light channels. A positive colocalization signal is considered indicative of adjacency of fluorophores and thus the molecules they label.

Plate cells on coverslips coated with poly-l-lysine (20 μ g/ml, 1 h, 37°C) and culture them (24 h, 37°C, 5% CO_2).

1. Wash cells in cold PBS and fix them with 4% paraformaldehyde (10 min, RT).
2. Treat the cells with cell-blocking buffer (1 h, 37°C) to avoid nonspecific binding.
3. Add the receptor-specific antibodies (30 min, RT). To facilitate the procedure, use antibodies of different origin (i.e., mouse, rabbit, rat, hamster) to stain the two receptors. The use of prelabeled antibodies precludes the need for secondary antibodies.

4. If necessary, add the mixture of prelabeled secondary antibodies (20 min, RT).
5. If both primary antibodies are of the same origin and isotype, add one of these antibodies (as in **step 4**), followed by its fluorochrome-labeled secondary antibody. Wash and incubate the cells with undiluted serum from the same species as the primary antibodies (30 min, RT). Now stain the cells with the specific antibody for the second receptor as in **step 4**, followed by its secondary antibody labeled with a different fluorochrome (20 min, RT).
6. Evaluate fluorescence on a confocal microscope using filters appropriate for the fluorochromes used (*see* **Note 10**) (**Fig. 1a**).

3.7. Resonance Energy-Transfer (RET) Techniques

There are two main types of RET, bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET). In the former, the donor molecule is luminescent (27, 28)

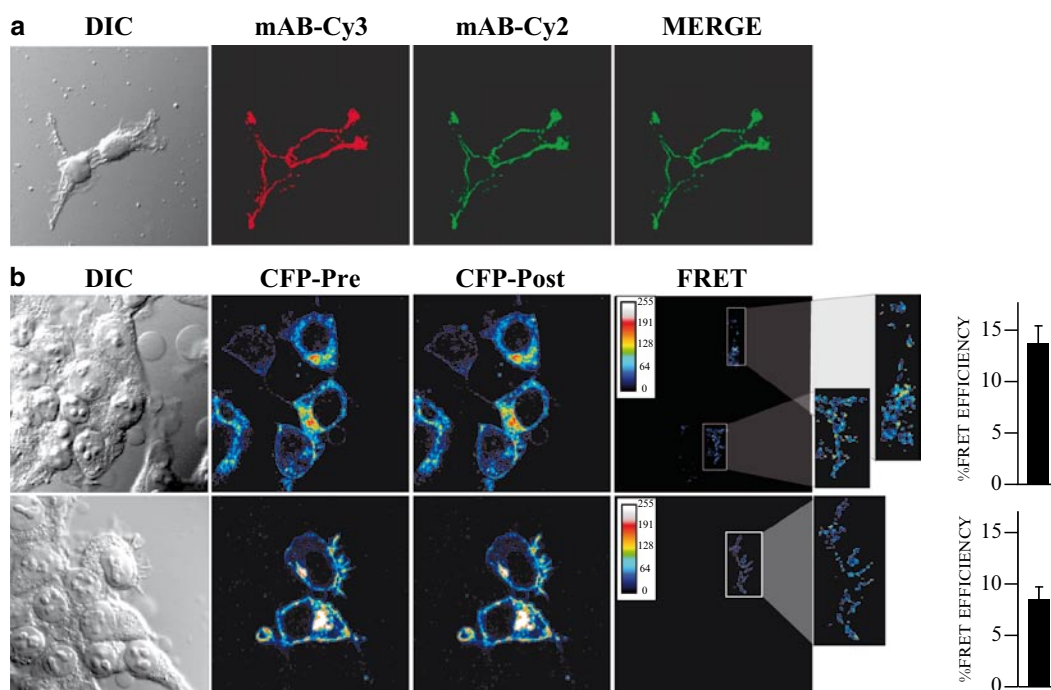


Fig. 1. Chemokine receptor localization and dimerization. **(a)** Chemokine receptors colocalize at the cell membrane. CXCR1/CXCR2 stably transfected HEK293 cells were fixed and costained with anti-CXCR1 and -CXCR2 mAb. A representative experiment shows staining of CXCR1 (anti-CXCR1-Cy3), CXCR2 (anti-CXCR2-Cy2), and the merged images (yellow). The DIC (differential interference contrast) image is also shown (left). **(b)** FRET evaluation of chemokine receptor heterodimerization using an acceptor photobleaching method. Unstimulated HEK293T cells were transiently cotransfected with combinations at a 1:1 ratio of (upper panels) CXCR5-CFP and CXCR5-YFP or (lower panels) CXCR5-CFP and CXCR4-YFP, fixed and FRET determined by photobleaching. A representative image is shown of CFP staining before (CFP-pre) and after (CFP-post) photobleaching; a false color merged image (FRET) and a zoom image of FRET at the photobleached area (inset) are also shown. Data are expressed as a percentage of FRET efficiency $\pm$ SEM. DIC images are shown for both cell types (left panels).

and in the latter, the donor fluorochrome transfers energy to an acceptor fluorochrome. Both techniques require the generation of fusion proteins between the receptor and the fluorescent/luminescent donor and acceptor proteins, as well as the use of transfected cells (29). Although BRET has been used at the single-cell level (30), it is in fact an approach for cell suspensions (31). This technique allows measurement of energy transfer between receptors independent of their expression pattern and permits quantitation. In contrast, FRET imaging techniques use confocal or wide field microscopy, allowing measurements in single cells and identification of cell locations at which FRET is detected.

BRET makes use of nonradiative energy transfer between a light donor and a fluorescent acceptor. In BRET, the bioluminescent energy resulting from the catalytic degradation of coelenterazine by luciferase is transferred to an acceptor fluorophore (for example, yellow fluorescent protein (YFP)), which in turn emits a fluorescent signal (32).

3.7.1. BRET Measurement

1. At 48 h post-transfection, wash cells once with PBS.
2. Add coelenterazine H to a final concentration of 5 μ M in PBS.
3. Collect readings using a multidetector plate reader that allows the sequential integration of signals detected in the 480 ± 20 nm and 530 ± 20 nm windows for luciferase and YFP light emissions, respectively (*see Note 11*).

For FRET, the donor emission and acceptor absorption spectra must have sufficient spectral overlap. As the donor excitation energy is transferred to the acceptor via an induced dipole-dipole interaction, efficiency depends on the distance between and orientation of donor and acceptor fluorophores (33). Ideal dyes are photo-stable, have little intensity fluctuation, and are relatively small in size, to minimize perturbation of the chemokine receptor.

Chimeric constructs can be generated in which each chemokine receptor is fused to a different, modified form of green fluorescent protein (GFP); the most commonly used variants are cyan (CFP) as donor and yellow (YFP) as acceptor (34). FRET is sometimes evaluated on intact receptors using specific pre-labeled antibodies. In this case, the use of secondary antibodies is occasionally necessary; although the increased distance between fluorophores complicates FRET detection, this can be resolved using dye-labeled F(ab') fragments. The most common dyes are Alexa488, FITC, Cy3, or Cy2 as donor and Rhodamine-2, Alexa555, Cy3, or Cy5 as acceptor (*see Note 12*).

Several methods are used to determine and quantify FRET.

1. Sensitized acceptor fluorescence. The donor fluorescent dye is excited and the acceptor signal is quantitated.
2. Acceptor photobleaching, a method based on quenching donor fluorescence. Some donor photons are used to excite

the acceptor, decreasing the emission energy detected. Photobleaching of the acceptor abolishes FRET, increasing donor light emission. This method cannot be used for living cells, however, since exposure to extended laser energy is thought to damage the cell.

3. Fluorescence lifetime imaging microscopy (FLIM) measures a chromophore's fluorescence lifetime, allowing spatial resolution of biochemical processes. The fluorescence lifetime of a donor dye decreases under FRET conditions, independent of fluorophore concentration or of excitation intensity (35).

3.7.2. FRET Measurement

1. Plate HEK293T cells (3.5×10^4 cells/ml) on poly-L-lysine-coated coverslips (20 $\mu\text{g/ml}$, 1 h, 37°C) and cultured them (24 h, 37°C, 5% CO_2).
2. Cotransfect receptor combinations at a 1:1 ratio (one YFP- and one CFP-labeled receptor) and culture them (48 h, 37°C, 5% CO_2).
3. Wash cells in PBS and fix with 4% formaldehyde (4 min, RT).
4. Wash 5 times in PBS and mount coverslips onto slides with PBS of pH 7.0 containing 80% glycerol.
5. Evaluate fluorescence on a confocal microscope using filters appropriate for the fluorochrome used (Fig. 1b).

In a typical FRET experiment, an image of the cell region of interest is taken using standard spectroscopic settings. CFP and YFP are excited with separate sweeps of the 405 nm (laser diode (25 mW at 12%)) and 515 nm lines (three-line argon laser (45 milliwatt maximum output, 6–10%)), respectively, and directed to the cell via a 405–440/515 nm dual dichroic mirror. Emitted fluorescence is split via an SDM 510-nm dichroic mirror for CFP, and directed to a spectral detector adjusted to the 460–500 nm range. For YFP, fluorescence is directed to a spectral detector adjusted to the 530–570 nm range. Confocal fluorescence intensity data (ICFPpre and IYFPpre) are recorded, with a pinhole of 100, as the average of four line scans per pixel and digitized at 12 bits. Repeated scans with 515-nm maximum light intensity are used to photobleach YFP, which requires 10–30 s at maximal scan rates and 100 pinhole aperture. After 60% of YFP bleaching, fluorescence intensity (ICFPpost and IYFPpost) is measured using identical parameters. FRET efficiency is determined on a pixel-by-pixel basis (E) and calculated in percent as $E = (\text{ICFP}_{\text{post}} - \text{ICFP}_{\text{pre}} / \text{ICFP}_{\text{post}}) \times 100\%$, where ICFPre and ICFPost are the background-corrected CFP fluorescence intensities before and after YFP photobleaching, respectively (36), using ImageJ 1.40g software (NIH). As a negative control, FRET should be determined in transiently transfected HEK293T cells with a chemokine receptor-CFP and in a region of the cotransfected cells without

photobleaching. FRET efficiency is calculated from three-five independent experiments using at least 50 images from each (*see* [Note 13](#) and [Note 14](#)).

3.7.3. Fluorescence Lifetime Imaging Microscopy (FLIM) Measurement

For FLIM determinations, cells are plated and transfected as in **Subheading 3.7.1**. FLIM measurements are performed using a confocal microscope with a High-Speed Lifetime Module and a 60× PlanApo 1.4 objective or equivalent equipment. Fluorescence lifetime is determined after excitation with a pulsed laser (picosecond pulses) and a bandpass emission filter appropriate for the fluorochrome used, and quantitated using LIMO (Nikon) or similar software. Avoid the use of mounting solutions, which increase autofluorescence.

3.8. Functional Assays

It is usually necessary to determine not only expression of a given receptor, but also its correct function and effector coupling. This is particularly important when using cells transfected with tagged or fluorescent protein-modified receptors, which although they are expressed correctly on the cell surface, can be nonfunctional.

3.8.1. Migration in Transwells

1. Select appropriate transwell pore size (usually 3, 5, or 8 μm) to allow chemotaxis but not random migration.
2. Resuspend cells (2.5×10^5) in 100 μl serum-free medium and add to the transwell insert.
3. Add the chemokines to the lower transwell chamber (in 0.6 ml serum-free medium).
4. Incubate (1–16 h, depending on cells used; 37°C, 5% CO_2).
5. Cells that migrate to the lower well are counted in a flow cytometer using a constant flow rate during fixed time periods (*see* [Note 15](#)). Controls include addition of chemokines to upper and lower transwell chambers to differentiate between ligand-induced chemotaxis and nonspecific migration; when possible, untransfected cells should also be used as controls.

Adherent cells usually attach to the filter and quantitation of migrated cells is therefore difficult, requiring direct counting by microscopy or cell detachment by protease cocktails. For example, some groups stain the filter with crystal violet after migration (0.5% in 20% methanol, 20 min, RT), and wash the filter repeatedly in distilled water until the background clears. They then scan cells and quantify by densitometry analysis of stained spots using ImageJ software. In these assays, linearity between cell numbers and densitometry quantification must be established for each cell type (*see* [Note 16](#)).

Other groups treat filters with trypsin/EDTA (10 min, 37°C) prior to quantitating detached cells using flow cytometry techniques. In both these cases, there is considerable variability in the data obtained, and several replicates should be used.

A commercially available 96-well plate-format migration chamber (Neuro Probe, Gaithersburg, MD) facilitates optimization and quantification of migration for adherent cells.

1. Mark the upper and the lower sides of the filter with a permanent marker for later orientation. Coat the filter with 10–20 ml of 20 µg/ml collagen IV or 5 µg/ml fibronectin and incubate (overnight, 4°C) with shaking. Allow filter to dry.
2. Detach cells in mild conditions (i.e., by incubating in 50 mM EDTA). To ensure that no clumps are formed, pipette the cells and maintain them with gentle shaking until use. Centrifuge ($300 \times g$, 7 min, RT) and resuspend cells in migration medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.25% BSA and 10 mM Hepes) at $1\text{--}3 \times 10^6$ cells/ml.
3. Fill the lower wells of the migration chamber with 30 µl/well of chemoattractant or agonist compounds in migration medium. Prepare quadruplicates for each condition. Pipettes must be perfectly calibrated. Work carefully but quickly.
4. Assemble the filter and rest of the migration chamber following manufacturer's instructions. Ensure that the medium forms a concave meniscus to avoid bubble formation when mounting the filter in the chamber. This is very important.
5. Fill the upper well with 200 µl of cell suspension and allow cells to migrate (4–6 h, 37°C). For example, when using HEK293 cells, use $1.5\text{--}3 \times 10^5$ cells in 0.2 ml migration medium.
6. Remove the cells remaining in the upper chamber wells by shaking the chamber over a sink or container. Remove the migration chamber thumb nuts and carefully remove the filter from the chamber.
7. Using a scraper, remove the cells remaining on the upper filter surface.
8. Stain the filter with crystal violet and quantify by densitometry analysis as earlier.

To reduce variability in migration values, other protocols include modifications to the protocols based on confocal microscopy technology.

1. Coat transwell filter with 20 µg/ml collagen IV or 20 µg/ml fibronectin (2 h, 37°C).
2. Label cells with CFSE, with 10^7 cells/ml in 1 µM CFSE (10 min, 37°C).
3. Resuspend cells (10^6 cells/ml) in serum-free medium and add 100 µl to the transwell insert.
4. Add the chemokines to the lower transwell chamber (in 0.6 ml serum-free medium).
5. Incubate 1–16 h, depending on the cells tested (37°C, 5% CO₂).

6. Fix the cells on the filter with 4% formaldehyde (10 min, RT).
7. Eliminate the cells remaining in the upper chamber wells and mount the chamber on the microscope to acquire images.
8. Acquire images using a 488-nm argon laser line and a 10× objective, with maximum pinhole aperture (800). Take 25 nonoverlapping predefined images to cover more than 65% of the total well surface.
9. Image fluorescence is quantitated using Image J64 software (**Fig. 2**).

3.8.2. Murine *in vivo* Cell Migration Assay

Migration is a multistep process that requires coordinated interaction between molecules on the surface of the migrating cells and receptors on the endothelial surface (37). Although some of these events can be mimicked *in vitro*, it is practically impossible to reproduce the coordinated action of several proteins (including integrins, selectins, chemokines) under flow conditions. Experiments should therefore be evaluated *in vivo*. At times, cells that have received distinct treatments are compared. In this case, cells must be labeled with vital dye cell trackers before beginning the migration experiment (*see Note 17*). For that, incubate cells (10^7 cells/ml) with cell tracker [calcein-AM (0.25 μ M, 5 min, 37°C, 5% CO₂), Cell Tracker Green (CMFDA, 0.3 μ M, 45 min, 37°C, 5% CO₂), or Cell Tracker Orange (CMTMR, 1.5 μ M, 45 min, 37°C, 5% CO₂)]. Following incubation, harvest cells by collecting with a pipette, centrifuge ($300 \times g$, 7 min, RT) and resuspend in PBS to 10^8 cells/ml.

Cell Migration to the Peritoneum

1. Inject the chemokine into the peritoneum (1 μ g in 100 μ l sterile PBS/mouse). Not all cell types migrate efficiently to the peritoneum in response to specific chemokines.

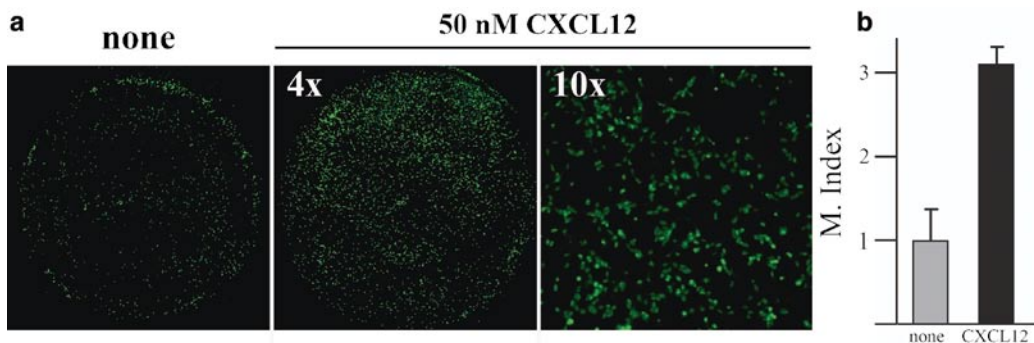


Fig. 2. Chemokines trigger specific cell migration. **(a)** CXCR4-expressing HEK293 cells were allowed to migrate toward a CXCL12 gradient (*center*) or medium as control (*left*). A representative image is shown of the filter using a 488-nm argon laser line and a $\times 4$ objective with maximum pinhole aperture (800) (*left, center*). A representative example shows one of the 25 predefined images captured with a $\times 10$ objective to cover the well surface (*right*); the migration index is calculated from these images. **(b)** The migration index is calculated using a fluorescence determination method and ImageJ software.

2. Allow endogenous cells to migrate for 4 h and kill mice by cervical dislocation or another IACUC-approved procedure.
3. Inject 2–5 ml of cold PBS into the peritoneal cavity of test mice and carefully recover the peritoneal lavage with a syringe. Repeat twice and combine all three lavages. Collect cells by centrifugation ($200 \times g$, 10 min, RT).
4. Label the cells with specific markers and quantify them by flow cytometry analysis.

Migration to the Spleen

1. Inject untreated or treated labeled cells into BALB/c mice (2×10^7 cells in 0.2 ml PBS, i.v.).
2. Anesthetize mice with ketamine (50 μ g/10 g body weight, i.p.) and xylazine (1 mg/10 g body weight, i.p.). Verify depth of anesthesia by loss of toe and tail pinch reflexes.
3. Shave the left side of the mouse and make a skin incision in the left superior quadrant of the abdomen. The spleen is easily identified as a dark red, elongated organ. Incise the peritoneum, expose the spleen and inject the chemoattractant or agonist test agents into it (1 μ g in 75 μ l sterile PBS/mouse). Close the peritoneal walls with suture and the skin with wound clips or skin staples.
4. Allow cells to migrate for 4 h and kill mice by cervical dislocation or other IACUC-approved procedure.
5. Remove the spleen and place it in a 100-mm Petri dish containing 10 ml of 37°C tissue culture medium. Tease apart the spleen using 40- μ m nylon mesh cell strainers until most cells have been released. Disrupt clumps by pipetting and transfer cells to a 15-ml centrifuge tube, leaving behind the larger pieces of tissue.
6. Lyse erythrocytes by adding 2 ml 0.85% ammonium hydroxide per spleen. Incubate (5 min, 37°C), then dilute with 20 ml tissue culture medium, and wash remaining cells by centrifugation ($300 \times g$, 7 min, RT) using culture medium.
7. Quantify the number of labeled cells by flow cytometry analysis as earlier.

Air Pouch Model

1. Anesthetize mice with ketamine/xylazine and verify depth of anesthesia as in **Subheading “Migration to the Spleen.”**
2. Inject 3 ml of air under the back skin. After 3 days, reinject the pouches with 3 ml of air.
3. On day 6, inject the chemokine or vehicle into the pouch.
4. Four hours after chemokine injection, anesthetize the mice as in point 1 and wash the pouches by injection and removal of 5 ml of saline solution.
5. Cool lavage fluid immediately on ice, and count endogenous cells by flow cytometry.

4. Notes

1. Heterologous expression systems are the preferred, and in some cases obligate models for study of GPCR oligomerization, although high expression levels can lead to nonspecific interactions.
2. Not all buffers preserve receptor association with signaling molecules or with other receptors. Many contradictory observations are easily explained by the use of different buffers.
3. Most antibodies can be obtained commercially. Although mAb offer unique specificity and reproducibility, polyclonal antibodies often have advantages for immunoprecipitation. The use of synthetic peptides as immunogens allows pre-determination of the receptor recognition target (for example, intra- or extracellular loops). If you are producing your own mAb, ensure that the screening criteria guarantee its use for the technique desired; an immunoprecipitating antibody will not necessarily work well for western blot or flow cytometry.
4. Immunoprecipitation and western blot allow the study a number of signaling events, including receptor dimerization and association between signaling molecules and receptors after ligand binding. Discrepancies between results in different studies are commonly attributed to detergent composition of buffers, which can disrupt some binding associations. Immunoprecipitation and western blot have been used successfully to determine receptor dimerization, for which a crosslinking step should be included prior to cell lysis. Resonance energy transfer (RET)-based techniques now provide a better analysis of dimerization events.
5. Stripping of nitrocellulose membranes is not recommended, as it can alter recognition of the transferred proteins.
6. Care should be taken in cell handling prior to the lysis step, as the presence of nonintact cells increases the background of nonspecific protein crosslinking. The bifunctional reagent solution should be prepared freshly.
7. Both selection of an appropriate tag and its location in the receptor are important factors. Amino acids added to the extracellular region can modify ligand binding, whereas modification of intracellular domains can disturb the coupling of signaling molecules. The analysis technique used will also dictate where the tag should be placed within the receptor. Flow cytometry analysis of intact cells requires tagging extracellular sequences, whereas western blot, immunoprecipitation, and immunofluorescence are less restrictive as to tag location. It is easy to construct and manipulate double-tagged receptors in a single cell; this method is useful for the study of receptor-receptor interaction; the main disadvantage is its limitation to transfected cells.

Receptor labeling circumvents the need to raise antibodies specific for target receptors, and has been used successfully to establish homodimerization of $\beta 2$ Ar_s, GABA_B, mGluR5, δ -opioid, m3-muscarinic, Ca²⁺ and CCR2 receptors (38). The position of the receptor tag must be chosen carefully so that receptor expression and ligand-binding properties are not affected. FLAG, myc, HA, GST, His, OMNI, MBP, S, Thio, GFP, CruzTag, and GAL are among the tags for which there are commercially available antibodies that immunoprecipitate and recognize the tagged receptor in western blot. Many vectors are available containing these tags, which allow their expression fused to the protein of interest. With this strategy, we used cells cotransfected with CCR2b receptor cDNA tagged in the N-terminal extracellular domain with Myc or YSKFDT (YSK) sequences, to show that the ligand induces receptor oligomerization (26).

The coimmunoprecipitation strategy is also used to detect heterodimerization, as receptor-specific antibodies allow tracking of heterodimerization not only in transfected cells, but also in cell lines or primary cells. We have used mAb specific for CCR2, CCR5, and CXCR4 in transfectants and primary cells to determine receptor heterodimerization (39). For such experiments, it is important to control assay specificity using a mixture of single transfected cells.

8. The construction of fluorescently labeled receptors uses standard molecular biology techniques. The fluorescent probe must be inserted in the C-terminal region to conserve the original signal peptide of the receptor. The fusion involves elimination of the receptor stop codon and insertion of the receptor into the fluorescent protein vector, separated by several additional amino acids. Transfect cells and analyze for correct expression and function.
9. Before use, Cy-labeled antibody should be titrated to determine the optimal dilution. To store the labeled antibody, mix it at a 1:1 ratio with glycerol and store in the freezer (−20°C).
10. A high-numerical aperture microscope lens permits resolution near 300 nm, sufficient to visualize the fluorescent label in different cell compartments, although not to demonstrate molecular association. Controls must be included to assure that the fluorescence of one fluorochrome does not overlap that of the other.
11. The BRET signal is determined by calculating the ratio of the light intensity emitted by receptor-YFP over the light intensity emitted by receptor-RLuc. The values are corrected by subtracting the background BRET signal as measured in the same cells expressing the receptor-RLuc construct alone.

For acquisition of full BRET spectra, cells are transfected with different amounts of receptor-YFP for a given quantity

of receptor-RLuc. Cells are detached and resuspended in PBS containing 0.1% (w/v) glucose. Cells (2×10^5) expressing different acceptor/donor (YFP/Luc) ratios are seeded in 100 μ l PBS in a 96-well plate with a clear bottom (Corning), and a BRET scan is performed by reading luminescence between 400 and 600 nm immediately after coelenterazine addition.

For BRET titration experiments, net BRET ratios are expressed as a function of the acceptor/donor ratio. Total fluorescence and luminescence are used as relative measures of total acceptor and donor protein expression, respectively. Total fluorescence is determined using an excitation filter at 485 nm and an emission filter at 535 nm. Total luminescence is measured 10 min after coelenterazine addition, in the absence of the emission filter.

12. Despite the many advantages of these techniques, their use raises several concerns. In most cases, RET requires cotransfection of donor and acceptor proteins, i.e., receptor overexpression, which may favor oligomer formation. In addition, receptor modification to incorporate donor and acceptor groups can alter receptor signaling. To avoid some of these problems, fluorescently labeled antibodies can be used with untransfected cells, although intracellular signaling molecules are not accessible in experiments using live cells.
13. For all these approaches, donor/acceptor choice is critical for FRET determination. Donor emission spectra should ideally have maximum overlap with acceptor absorption spectra, although acceptor and donor emissions should be clearly separable to minimize background interference. Fluorochrome incorporation into the protein to be analyzed must also be considered. The most appropriate donor/acceptor combinations are blue (BFP)/GFP, CFP/YFP, green (GFP)/dsRed, and YFP/dsRed. In practice, the CFP/YFP combination is the most frequently used, as both molecules are extremely bright and this combination offers few technical problems. BFP is a poor donor as it is not especially bright, making FRET between BFP and GFP difficult to detect. dsRed is a poor acceptor, as it has a broad absorption spectrum and excites the same wavelength as the donor (GFP or YFP). In addition, constructs that work well with GFP and its variants do not work using dsRED. Specific antibodies conjugated to appropriately selected fluorescent dyes can be used to study fixed cells. For example, although Cy3 as donor and Cy5 as acceptor form a suitable fluorochrome pair, the Cy2 donor/Cy3 acceptor pair is often more convenient, as it permits use of the widely available 488 nm argon laser line. Secondary antibodies are occasionally necessary, although the increased distance between fluorophores complicates FRET detection; this can be resolved using dye-labeled F(ab') fragments.

14. As FRET depends on fluorochrome distance and orientation, low FRET efficiency values do not necessarily correlate with lack of dimerization. If the role of the ligand on receptor dimerization is required, cells should be stimulated before to fix them.
15. To obtain a direct determination of migrated cells, 10 μ l of fluorescent microspheres (Flow-Count fluorospheres; Beckman Coulter, Fullerton, CA) are usually added to the medium before flow cytometry analysis as a standard.
16. Cell numbers required per well vary greatly from one cell type to another and should be precalibrated.
17. Cell trackers and calcein AM are membrane-permeable fluorescent probes that can be used to visualize live cells. Cell Tracker probes are fluorescent chloromethyl derivatives that are processed into membrane-impermeable compounds retained in cells for up to 72 h after loading, and that can be passed to daughter cells. The live cell indicator calcein AM is a nonfluorescent, cell-permeable compound that is hydrolyzed by intracellular esterases into the fluorescent anion calcein. The cell tracker concentration should be calibrated for each cell type.

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Chapter 13

Intravital Two-Photon Imaging of Adoptively Transferred B Lymphocytes in Inguinal Lymph Nodes

Chung Park, Il-Young Hwang, and John H. Kehrl

Summary

Intravital two-photon imaging allows the observation of immune cells in intact organs of live animals in real time. Recently, several studies using two-photon microscopy have detailed the motility of mouse B and T lymphocyte within lymph nodes and have shown a dependence upon chemokine receptor signaling for the basal velocity of the cells. For, example, T cells from *Gnia2*^{-/-} mice, deficient in the heterotrimer G-protein G α subunit G α_{i2} have markedly impaired chemokine-triggered chemotaxis. In vivo these cells have reduced motility and impaired positioning within lymph nodes. *Gnia2*^{-/-} B cells exhibit similar defects. In addition, B cells from *Rgs1*^{-/-} mice, deficient in a major negative regulator of G α_i , have a more robust motility than do wild-type B cells. Here, we describe procedures for visualizing the behavior of fluorescently labeled and adoptively transferred B lymphocytes within the inguinal lymph node of live mice.

Key words: B lymphocytes, Inguinal lymph node, Intravital imaging, Two-photon microscopy

1. Introduction

Chemoattractants and their receptors precisely orchestrate the primary migration of developing lymphocytes into and out of secondary lymphoid organs (SLOs) and the organization of second immune anatomic structure within the SLO such as B cell follicles and germinal centers (1–3). Many draining lymph nodes (LN) are located at sites superficially under the skin. They are well-compartmentalized structures with specific microenvironments for lymphocytes. Many lymphocytes reside only temporarily within lymph nodes since they continually recirculate between the blood and the lymph. Most human and mouse lymphocytes enter

lymph nodes from the blood (a minority via afferent lymphatics) and exit lymph nodes using the efferent lymphatics before returning to the blood via the thoracic duct (4–7). Recently, the use of highly advanced two-photon microscopy technology has allowed real-time imaging of adoptively transferred, fluorescently labeled lymphocytes in the superficial lymph nodes of live animals (8–11). Several imaging studies have provided a basis for arguing that chemokine receptor signaling contributes to the basal motility of lymphocytes within lymph nodes (12–14).

Adoptively transferred B lymphocytes home rapidly to lymph nodes. Within two hours of transfer many B cells will exit the blood through high endothelial venules, enter the lymph node cortical ridge region, and then move into the lymph node follicles (13). Because the commonly used two-photon lasers can easily reach 200–300 μm beneath the lymph node surface intravital imaging of B cell follicles, most of which are located between 30 and 300 μm under capsule of lymph node, is readily achievable (15). Adoptively transferring both B and T lymphocytes readily allows the discrimination between the B and T cell zones in the lymph node during imaging. A critical control for these types of experiment is the concurrent transfer of wild-type or nontreated cells labeled with another fluorophore. Control and experimental cells can then be imaged simultaneously and their behaviors directly compared. A major consideration in intravital microscopy is the surgical procedure required to expose the inguinal or popliteal lymph node of the anesthetized mouse prior to imaging. It is important not to damage the tissues surrounding the lymph node so as to not disturb normal physiological conditions. Particular care is needed not to disrupt lymph node innervation or the normal flow of blood and lymph. The intravital imaging procedure detailed later is for imaging the mouse inguinal LN and is a modification of a previously published method (13). It can be adapted to imaging the popliteal LN.

2. Materials

2.1. Preparation of B Lymphocytes from Mouse Spleen

1. 1 $\times$ Phosphate-buffered saline (PBS), pH 7.4
2. RPMI-1640 medium (Gibco)
3. Fetal calf serum (FCS), quantified (Gibco)
4. 40 and 100 μm of Nylon Cell strainer (BD Falcon)
5. 25 $\times$ 5/8 – gage needles (Kendall)
6. 12-mL syringe (Kendall)

7. ACK lysis buffer (BioWhittaker, Lonza)
8. Bovine serum albumin (BSA) fraction V.
9. Biotinylated mouse-Ab (MAb) (BD Pharmingen)
10. Magnetic negative selection systems: Dynabeads and magnetic particle concentrator (Invitrogen)
11. Fluorescence-activated cell sorting (FACS) buffer: 1× PBS, pH 7.4, 1% BSA.

2.2. Labeling B Lymphocytes and In Vivo Transfer of Labeled-B Lymphocytes

1. CellTracker™ probes (Molecular Probes, Invitrogen) for long-term tracing of living cells: 5-Chloromethylfluorescein diacetate (CMFDA) and 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR), stock solution 5 mM in DMSO (stored at -20°C). 4-Chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF₂HC), stock solution 50 mM in DMSO (stored at -20°C).
2. RPMI-1640 medium (Gibco).
3. Fetal calf serum (FCS), quantified (Gibco).
4. 1× Phosphate-buffered saline (PBS), pH 7.4.

2.3. Multiphoton Imaging

1. Two-photon laser-scanning microscopy system. We use a Leica SP5 inverted 5 channel confocal microscope (*see* [Note 1](#) and [Fig. 1a](#)), a Ti:Sapphire laser (Spectra Physics) with a 10-W pump tuned to 810 nm, a 20× multi-immersion objective with long working distance (for example, Leica 20× 0.7NA), an incubation cube chamber (Life Imaging Services, Basel, Switzerland) (*see* [Note 1](#) and [Fig. 1b](#)).
2. Heating Mantles & Blankets (Thermo Scientific). The heating blanket is set up to heat at the 37°C .
3. Anesthesia: 50× Avertin (tribromoethanol and 2-methyl-2-butanol, Sigma-Aldrich), stored at -20°C . Diluted prior to use; see later.
4. Small animal clipper (Fisher Scientific).
5. *Microsurgical instruments*. Roboz surgical scissor and forceps.
6. Cover-glass chamber slide (Nalgene, Nunc).
7. Classic Double Edge Blades 5-Pack (Wilkinson Sword).
8. 1× Phosphate-buffered saline(PBS), pH 7.4.
9. Cyanoacrylate glue.
10. Infusion set (Butterfly ST, 25× 3/8 32/2 in. Tubing).
11. Dextran conjugates (dextran, Alexa fluor 488, -594, cascade blue; 10,000 MW, anionic, fixable) (Molecular Probes, Invitrogen).

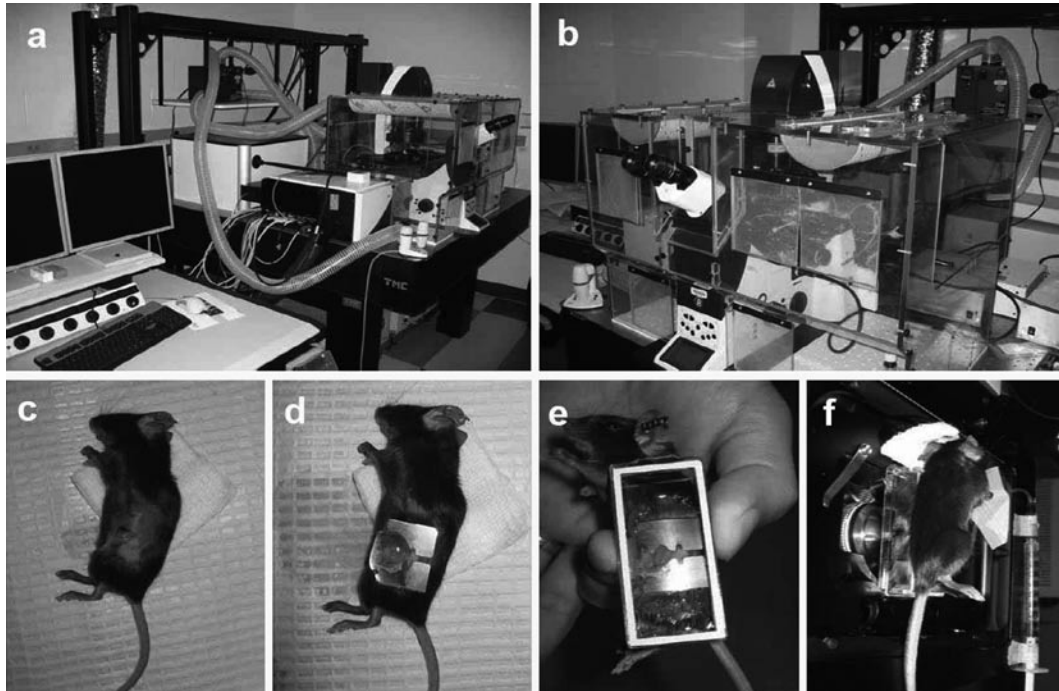


Fig. 1. Two-photon laser-scanning microscopy system and operation for exposing the inguinal lymph node. (a) Two-photon laser-scanning microscopy system including a Leica SP5 inverted 5-channel confocal microscope. (b) The cube and box, cube chamber, maintains temperature at $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. (c) Anesthetized mouse is shaved and has small incision on flank. (d) Immobilized iLN with cyanoacrylate glue on blade fitted to chamber slide. To protect tissue from drying add a few drops of PBS to exposed iLN. (e) Mouse in a warmed chamber slide. Blade can hold exposed iLN preventing respiratory movements. (f) Complete set up for imaging. Mouse is anesthetized by the intraperitoneal injection of avertin with an infusion set.

3. Methods

We described details of procedures for real-time imaging of adoptively transferred B cells visualized with fluorescent dyes in the inguinal lymph node. This method can also be used to observe behavior of other immune cells (T lymphocytes, dendritic and follicular dendritic cells, etc.) under a variety of experimental conditions.

3.1. Preparation of B Lymphocytes from Mouse Spleen (See Note 2) (16)

1. Mice are killed and their spleens are harvested.
2. The spleens are disrupted with forceps or needles in $1\times$ PBS (PBS) and the cells dissociated by gentle teasing followed by filtering through a 100- and then a 40- μm nylon cell strainer to remove connective tissue cells.
3. Splenocytes are depleted of red blood cells with ACK lysis buffer and subsequent washing with PBS.

4. The cell suspensions are centrifuged at $300 \times g$ for 5 min at 4°C , the supernatants removed, and the pellet resuspended in FACS buffer.
5. A count of the suspensions is made to determine cell number per milliliter (mL).
6. Cells are pelleted and resuspended in FACS buffer. Cells are stained with biotinylated monoclonal antibodies (MAb) to non-B-cell markers such as CD4, CD8, GR-1, Mac-1, Ter119, CD11c, and DX5 in FACS buffer (*see Note 3*).
7. Cells are incubated at 4°C for 15 min and washed with FACS buffer.
8. Pelleted cells are resuspended in FACS buffer. Dynabeads M-280 streptavidin beads are washed with PBS twice and with FACS buffer twice. The bead-to-cell ratio is determined by manufacturer's protocols (*17*).
9. The washed magnetic beads are added to the cell suspensions and incubated at 4°C , slowly rotating for 15 min. The suspensions are then attached to the magnet and allowed to separate.
10. The nonadherent suspension is collected and reapplied to the magnetic source, and again the nonadherent cells are collected: This population is washed, counted, resuspended in RPMI/10% FCS, and incubated at 37°C for 30 min prior to any stain.

3.2. Labeling B Lymphocytes and In Vivo Transfer of Them

1. Resuspend $0.5\text{--}10 \times 10^6$ B lymphocytes/mL in RPMI/10% FCS with $1\text{--}5 \mu\text{M}$ CMFDA and CMTMR or $20\text{--}50 \mu\text{M}$ CMF₂HC dye (*15*), respectively. Incubate the lymphocyte for 20 min at 37°C and wash five times with PBS (*see Note 4*).
2. Resuspend labeled B lymphocytes in PBS and inject intravenously into lateral tail vein of recipient mice.
3. Intravital imaging is best performed 12–24 h after injection although B cells crossing high endothelial venules and entering the cortical ridge region of the inguinal lymph node can be visualized within minutes of adoptive transfer.

3.3. Anesthesia

1. Make 50× Avertin (76.9%) stock by dissolving 5 g Tribromoethanol in 6.5 mL of 2-Methyl-2-butanol.
2. Solution must be filtered with a $0.22\text{-}\mu\text{m}$ syringe filter (Milipore) before making aliquots of the solution in 1-mL stock vials. Can be maintained at -20°C for 1 year.
3. To make 1× Avertin (1.51%), mix 200 μL of 50× Avertin stock with the 10 mL prewarmed PBS. This solution can be stored at 4°C for 2 weeks.

4. Inject the mouse peritoneum with 1× Avertin at a dose of 300 mg/kg. Check the level of anesthesia by monitoring body and respiratory function (9).

3.4. Operation for Exposing the Inguinal Lymph Node (iLN)

For all the following steps, the mouse should be transferred onto the heated blanket. Inguinal lymph nodes are prepared for intravital microscopy using a modification of a published method (13).

1. After shaving the hair of the left or right flank with a small animal clipper, the skin and the fatty tissue over the inguinal lymph node are removed (*see Fig. 1c*).
2. To visualize both B cell follicle and lymphatic area in the same imaging field, the exposed inguinal lymph node must be turned 90° from its original direction.
3. The mouse inguinal lymph node is held with a razor blade fitted to coverglass chamber slide (*see Fig. 1d*).
4. The mouse is placed in a prewarmed coverglass chamber slide with 2 mL prewarmed PBS at 37°C is added (*see Note 5 and Fig. 1e*).
5. The chamber slide is then placed into the temperature control cube chamber equipped on Leica SP5 microscope systems (*see Note 1 and 5 and Fig. 1d*). Glycerol (70% in H₂O) is used as an immersion fluid.

3.5. Imaging using Multiphoton Microscopy (See Fig. 1)

1. Tune the Mai Tai Ti:Sapphire laser to 800–830 nm.
2. For four-dimensional analysis of cell migration, set up the microscope configuration and filter set for the detection of the following dyes (CMFDA, CMTMR, CMF₂HC, and second harmonics) (*see Note 6*).
3. Using mercury lamp illumination, look through the eyepiece and focus onto the surface of the iLN.
4. Start scanning using the Mai Tai Ti:Sapphire laser (*see Note 7*). Adjust gain and offset of the detectors. Typically, labeled B cells in B cell follicles can be detected 30 μm from the capsule. While scanning, move the *x*-, *y*-, and *z*-axes to locate an area containing a sufficiently high density of B cells.
5. A suitable location is found, acquire *z* stacks of optical sections (typically 11 or 17 sections spaced by 5 μm) every 15 or 30 s for a total duration of 20–30 min.
6. Check the level of anesthesia before starting a new image acquisition.

3.6. Analysis

1. A sequence of image stacks is transformed into volume-rendered four-dimensional movies using Imaris software (Bit-plane) (*see Fig. 2*).

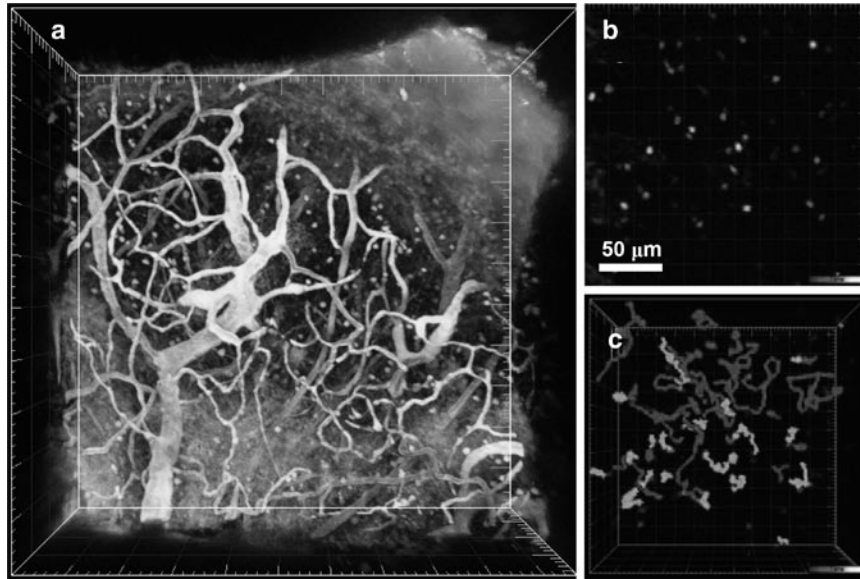


Fig. 2 Reconstituted images of intravital images and example of analysis. (a) Maximum projection of a Z-stack (200 μm) image composed from images taken every 0.5 μm . Blood vessels were visualized by an intravenous injection of dextran conjugates. (b) Two-photon intravital imaging of inguinal lymph node. Reconstructed 3D structure (70 μm Z-projection) shows adoptively transferred B lymphocytes (*light gray* (CMFDA; *Green*) and *dark gray* (CMTMR; *Red*)). (c) Trajectory of transferred B lymphocytes tracked with Imaris in the 3D volume.

2. The spot analysis is used for semiautomated tracking of cell motility in three dimensions by using the parameters of autoregressive motion as an algorithm using Imaris.
3. Calculation of cell velocity and displacement is performed using Imaris following the manufacturer's instructions.
4. Our statistical analysis and significance of static's calculation are performed using GraphPad Prism (GraphPad Software).
5. QuickTime movies can be generated following a maximum intensity projection of each Z stack. Editing and labeling of the resulting movies can be performed with Adobe Premier Pro CS3 program (Adobe).

4. Notes

1. It is recommended that nondescanned detectors be used for collecting even very weak fluorescent signals. Air temperature in cube chamber (The Cube and Box) (Life Imaging Services) should be monitored and maintained at $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

Temperature control is very important for detecting proper cell movement (4).

2. B lymphocytes can be purified from bulk population by removing non-B-lymphocytes. There are multiple methods to achieve this purification; this subheading describes one such procedure (refer to methods published by Dynal, Inc., Miltenyi Biotec-MACS).
3. The indicated biotinylated MAbs will bind CD4 & CD8 T cells, macrophages, erythrocytes, NK cells, granulocytes, and dendritic cells.
4. When staining low cell concentrations FCS can help protect cells from the toxic effects of the dyes.
5. Inguinal lymph node is maintained at physiological temperature because lymphocyte motility and behavior are highly temperature dependent (8).
6. Emitted fluorescence is collected using a 4-channel nondescanner detector. Wavelength separation is through a dichroic mirror at 495 nm and then separated again through a dichroic mirror at 455 nm followed by 405/20 nm emission filter for second harmonics and 480/40 nm emission filter for CMF₂HC; a dichroic mirror at 565 nm followed by 525/50 nm emission filter for CMFDA and 620/60 nm emission filter for CMTMR.
7. Scrupulous care should be taken to minimize the laser power used for excitation as excessive power can cause phototoxicity resulting in cell movement arrest (18).

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Chapter 14

Breast Cancer Cell Movement: Imaging Invadopodia by TIRF and IRM Microscopy

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Summary

Invadopodia are hair-like membrane protrusions projecting from the ventral side of the plasma membrane of tumor cells invading into an extracellular matrix (ECM). Formation of invadopodia and phagocytosis of partially degraded ECM is correlated with invasiveness of cancer cells. Many proteins associated with actin-rich punctae associated with invadopodia have been identified. However, the dynamic temporal and spatial relationship of invadopodia-related proteins and the mechanisms required for invadopodia formation remain largely unknown. Total Internal Reflection Fluorescence (TIRF) microscopy provides a powerful tool to directly visualize the dynamic membrane transportation of invadopodia-related, GFP-tagged proteins and to simultaneously monitor invadopodia formation by observation of localized degradation and phagocytosis of fluorescently labeled gelatin. Cell-substratum contacts can be visualized using a related technique, Interference Reflection Microscopy (IRM). In this chapter, we provide detailed methodologies to monitor the dynamic localizations of c-Src-eGFP using two-color TIRF microscopy along with IRM to simultaneously visualize translocation of c-Src-eGFP and invadopodia formation by degradation of AlexaFluor® 568-labeled gelatin.

Key words: Breast cancer cell migration, invasion, invadopodia, Total Internal Reflection Fluorescence (TIRF) microscopy, Interference Reflection Microscopy (IRM)

1. Introduction

Chemoattractant-induced cell migration is carried out by the protrusion of the cell membrane. Protrusive structures have different morphological and functional characters (1, 2). Cells migrating on a surface of substrates (2D) form protrusions called filopodia and lamellipodia at the leading edge. Cells entering a dense extracellular matrix, such as tumor cells migrating into tissue, form additional protrusions thought to be invadopodia. Invadopodia were first characterized by and functionally referred to as the localized degradation of ECM by membrane-associated proteinases (3–5).

Invadopodia are hair-like structures that protrude from the ventral side of the plasma membrane into ECM at these localized sites of degradation and invasion (4). Invadopodia formation is frequent in invasive cancer cells, including primary human tumor cells (6–9); thus, we believe that invadopodia represent a universal organelle for cancer cell invasion. It is hypothesized that breast cancer cells utilize invadopodia to migrate through tumor stroma and into blood vessels in the process of metastasis, although this has not been formally proven.

Many proteins are associated with invadopodia and can be classified into four major functional groups (10). The first group includes motility-related proteins including actin and regulators of filamentous actin (F-actin), such as Arp2/3, N-WASP, Cdc42, Nck, cofilin, actin-capping proteins, cortactin, and dynamin. The second group includes adhesion proteins, such as multiple integrins, that mediate interaction between invadopodia and ECM. The third group comprises signaling proteins that regulate the actin cytoskeleton and membrane remodeling, such as tyrosine kinases and Ras-related GTPases. Membrane-associated proteinases, mediating ECM degradation at the sites of invadopodia, constitute the fourth group. Among these are membrane type 1 matrix metalloproteinase (MT1-MMP), seprase, MMP-2, and the urokinase-type plasminogen activator (uPA)/uPA receptor proteolytic system (11). The molecular mechanisms of invadopodia formation and their biological function, however, are not fully understood.

One striking feature is that invadopodia typically form underneath the cell body, often close to the nucleus and proximal to the Golgi complex, which is the central station in intracellular trafficking events (12). Multiple lines of evidence connect vesicular trafficking pathways to invadopodia formation and function (11, 13, 14). It seems that phagocytosis may be the major mechanism for uptake of ECM (7, 15). In the reverse direction, secretion of invadopodia components is an attractive mechanism for the targeting of molecules such as proteases to sites of ECM degradation. Invadopodia formation has been assessed by *in vitro* fluorescent matrix degradation assays (16, 17). We have previously implemented the use of fluorescent, crosslinked gelatin to detect sites of invadopodia-associated proteolytic activity, previously described by Chen et al. (3) and further modified this assay for detection of phagocytosed partially degraded gelatin using fluorescence-activated cell sorting (FACS) (7). Considerable information on the requirement of individual proteins for invadopodia function has been gleaned by quantification of invadopodia and phagocytosis by these techniques after transfection, knockdown, peptide or pharmacological inhibition. The penetration of invadopodia vertically into the crosslinked layer has been visualized by us and others using confocal microscopy and multicolor detection of combinations of invadopodia-related proteins to determine their spatial relationship to one another. However, the fine structure

of the invadopodia, the abundant cytosolic and perinuclear distribution of many of these proteins, and the limitations of vertical resolution using confocal microscopy make it difficult to image the dynamic process in live cells.

Total Internal Reflection Fluorescence (TIRF) microscopy provides a unique tool to simultaneously monitor protein transportation to the plasma membrane and invadopodia formation. TIRF microscopy applies an optical phenomenon called total internal reflection, that is, when light travels between the interface of two media with different refractive indices (going from a higher to lower refractive media), the light incident at an angle greater than the critical angle undergoes total reflection. Beyond the angle of total reflection, the electromagnetic field of the incoming/reflected light still extends into the Z direction, termed an evanescent field. One property of the evanescent wave is the strength of the evanescent wave decreases greatly with distance, and its effects extend only a few hundred nanometers into the second medium. Only the portion of the specimen within the evanescent field, usually cell attachment sites, can be excited to emit fluorescence and consequently can be seen or recorded. TIRF microscopy provides a direct way to observe events occurring on the cell membrane, such as membrane vesicle transportation, with greatly decreased cytosolic “noise.” In this chapter, we focus on introducing the methodology of applying TIRF microscopy along with interference reflection microscopy (IRM) which is used to visualize cell-substratum points of contact, to simultaneously monitor protein transportation and invadopodia formation.

2. Materials

2.1. Cell Culture

1. IMEM medium (Cellgro, Mediatech Inc, Manassas, VA) supplemented with fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA).
2. Trypsin.
3. L-Glutamine, (Gibco, Invitrogen Life Science, Bethesda, MD, Cat# 25030).
4. Geneticin[®], a stock concentration of active 50 mg/mL, is stored at 4°C (Gibco, Invitrogen Life Science #10131-027). This pre-tested stock, although more expensive, is used to save the step of determining the activity of untested solutions of antibiotic.
5. T25 culture flasks (BD Biosciences).
6. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution is frozen at -20 °C in aliquots (Gibco, Invitrogen Life Science); add 5 mL to 0.5 L of IMEM complete culture media.

7. Cell lines:
 - (a) BT549 cells stably expressing PKD1-eGFP.
 - (b) BT549 cells stably expressing c-Src(Y527F)-eGFP.
8. Cell culture incubator, 37°C, 5% CO₂.

2.2. Labeling Gelatin with AlexaFluor 568

1. *Sterile 1× PBS*. 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.2.
2. 2 mg/mL Gelatin (Sigma-Aldrich) in PBS. Store as 0.5 mL aliquots at −20°C for up to 1 year (*see* [Note 1](#)).
3. 1 M Sodium azide in deionized water, stored at 4°C for up to 2 months.
4. AlexaFluor 568 protein labeling kit (Molecular Probes, Invitrogen Life Sciences).
5. Sodium Bicarbonate.

2.3. Preparation of MatTek Dish Coated with AlexaFluor 568-Labeled Gelatin

1. AlexaFluor 568-gelatin (~1 mg/mL after purification in **Sub-heading 3.2.2**).
2. Poly-l-lysine, (Sigma), 1 mg/mL stock in 4°C good for years.
3. 0.5% Glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS, stored at 4°C for up to 1 month.
4. MatTek dish, #1.5 coverslip, uncoated, 35 mm (MatTek Corporation, Ashland, MA).
5. IMEM serum-free media (Cellgro, Mediatech Inc.).
6. *70% Ethanol*. Dilute USP-grade ethanol with deionized water; store at room temperature.

2.4. Imaging with Olympus TIRF Microscope

1. Olympus microscope IX81F2 (Olympus America, Inc., Center Valley, PA).
 - (a) Motorized fluorescence 6-cube turret.
 - (b) Plan Apo 60× OIL/ N.A. 1.45, WD 0.1 mm with correction collar (#PLAPON 60xO TIRFMSP 1.45, Olympus America, Inc.).
 - (c) L-shaped fluorescence condenser, with centerable aperture stop (AS), field stop (FS), and filter slider (#IX2-RFAL, Olympus).
 - (d) IRM and TIRF are split at the epifluorescence port (for example, *see* [Fig. 1c](#) in ref. [18](#)) (#IX2-RFAEVA-2, Total Internal Reflection Fluorescence illuminator for IX2 and IX frames. Dual light illumination system, both with field stop and ND slider, Olympus).
 - (e) Electron Multiplier CDD camera C9100-12 512X512 (Hamamatsu Corporation, Bridgewater, NJ).

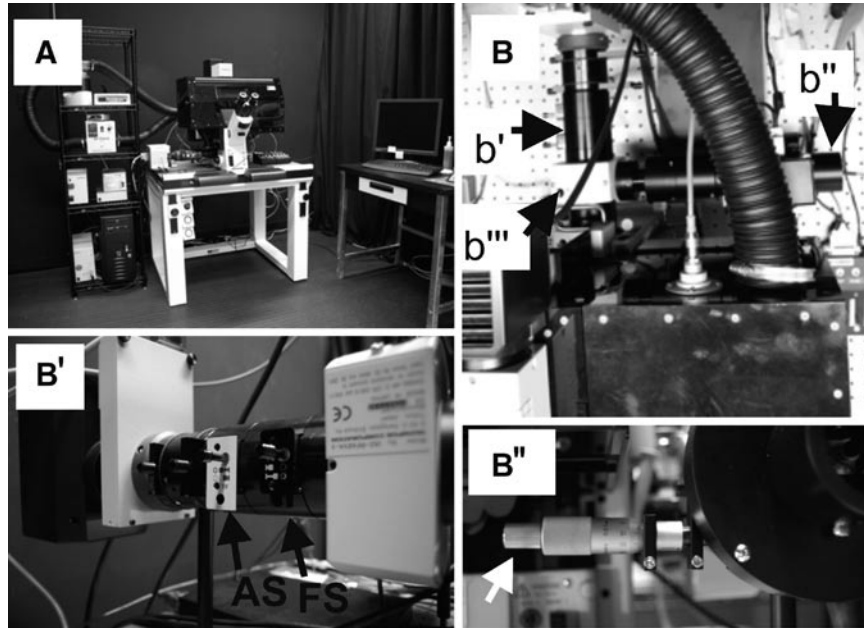


Fig. 1. TIRF microscope. (A) A view of the TIRF system. (B) View looking down on the back of the microscope (environmental chamber at bottom, back of the system at the top). Arrow b'' indicates the location of the Selection Prism containing the 80%/ 20% beamsplitter. Pulling the knob at the top to its full upwards position sets the beamsplitter to 100%/ 0% (all Epi-illumination input to the microscope). Pushing it downward sets the beamsplitter to 80% laser and 20% epifluorescence illumination. (B') Location of the Field Diaphragm and the Field Stop on the epifluorescence arm of the split box. Two arrows AS and FS indicate Aperture Stop and Field Stop, respectively. Adjustment of these is vital for good IRM imaging. (B'') The laser input from which the screw (white arrow) for TIRF angle adjustment projects. This pair of screws laterally translocates the laser path off center in the objective so that the angle of reflection is altered.

- (f) Micro-Imager DUAL-View (Optical Insights, LLC, Santa Fe, New Mexico) on the left side port between the microscope and the camera.
- (g) Microscope-enclosed environmental chamber for temperature and CO_2 control (Olympus).
- (h) Temperature control module (World Precision Instruments, Sarasota, FL).
- (i) CO_2 sensor (Biospherix Pro: CO_2 controller, sensor model GMP221mono S/N A3020005, Lacona, NY).
- (j) Humidifier (KAZ, Southborough, MA).
- (k) Software, IPLab4.0 (BD Biosciences Bioimaging) for Windows with motion control and Multiprobe. Alternates are Metamorph ver. 7.5 Acquisition software (Molecular Devices) and Slidebook 4.2 (Olympus).
- (l) Olympus immersion oil (Olympus).
- (m) Lens cleaning paper (Olympus, Whatman 105).
- (n) Mercury lamp (100 W mercury discharge lamp #U-RFL-T, Olympus).

2. TIRF design.

- (a) Krypton–Argon laser (568 nm, 647 nm, 100 mW total output, polarized); Argon-ion laser (457 nm, 488 nm, 514 nm, 200 mW total output, polarized) (Prairie Technologies, Inc, Middleton, WI); laser cleanup filters (457 nm, 488 nm, 514 nm, 568 nm, 647 nm).
- (b) Single-mode optical fiber with laser couple (458–633 nm), 5-UR478. IX2-RFAEVA2-TIRFM fiber illuminator class 3B.
- (c) *Achromat Doublets*. 100 mm EFL, 26.5MM.DIA Achromat, Cat# DBL14026/101; 200 mm EFL, 30MM DIA Achromat, Cat# DBL14133/101 (JML Direct Optics, JML Optical Industries, Inc., Rochester, NY) (*see* [Note 2](#).)
- (d) Acoustic Optic Tunable Filter (AOTF) tuned to 442 nm, 457 nm, 488 nm, 514 nm, 568 nm, 647 nm (Prairie Technology).

3. IRM/epifluorescence design

- (a) Neutral density filters. (2.0, 1.3, 1.0, 0.6, 0.3 filters).

3. Methods

3.1. Cell Culture

1. BT549 cells stably expressing PKD1-eGFP and c-Src(Y527F)-eGFP are cultured in IMEM complete media containing IMEM medium, 10% FBS, and 2 mM glutamate. To maintain the expression of eGFP-tagged proteins, Geneticin at a final concentration of 2 mg/mL is added to the cell culture each time the cells are passed.
2. Cells are passed every 2 or 3 days.

3.2. Preparation of AlexaFluor 568-Labeled Gelatin

*3.2.1. Labeling Gelatin
Using AlexaFluor 568
Protein Labeling Kit*

This protocol is slightly modified from that provided with the kit.

1. Prepare a 1 M solution of sodium bicarbonate by adding 1 mL of deionized water to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved.
2. Allow a vial of reactive dye to warm to room temperature.
3. Add 50 μ L of 1 M sodium bicarbonate to 0.5 mL of 2mg/mL gelatin to adjust its pH to ~8.3.
4. Transfer 0.5 mL of the gelatin solution to the reactive dye vessel. Invert a few times to fully dissolve the dye. Stir the reaction mixture for 1 h at room temperature in the dark using a magnetic stirrer and the stir bar already provided in the vial.

3.2.2. Purification of the Labeled Gelatin

1. Assemble the column and position it upright (*see* [Note 3](#)).
2. Prepare elution buffer by diluting the provided 10× stock to 1× in deionized water. Typically, less than 10 mL will be required for each column purification.
3. Gently resuspend the purification resin and if three columns will be run simultaneously, divide it equally into three beakers. Attach the provided funnel to the column and carefully and evenly pour one-third of the resin into the column to form a densely packed resin bed. Allow the column to flow until the elution buffer stops at the top of the column (*see* [Note 4](#)).
4. Carefully load the reaction obtained in **step 4** of **Subheading 3.2.1** onto the column and rinse the reaction vial with 100 µl elution buffer and also apply to the column (*see* [Note 5](#)).
5. After the sample has completely entered the column, slowly add elution buffer. Be careful not to disturb the column bed. Continue adding elution buffer until the labeled protein has been eluted (typically about 30 min).
6. As the column runs, periodically observe the separation. You should observe two fluorescent bands: a lower AlexaFluor 568-labeled gelatin band and an upper (slower) unlabeled dye band. Use the vial provided to collect the lower band.

3.3. Preparation of MatTek Dishes Coated with AlexaFluor 568-Labeled Gelatin

1. Warm AlexaFluor 568-labeled gelatin obtained in **Subheading 3.2.2** and 0.1% gelatin in 38°C water bath.
2. Treat MatTek dish with 0.4 mL of freshly made 12.5 µg/mL poly-l-lysine in distilled water for 20 min at room temperature (*see* [Note 6](#)).
3. Wash dish 3 times with 1 mL of PBS each time.
4. Treat MatTek dish with 0.4 mL of 0.5% glutaraldehyde for 15 min at room temperature (*see* [Note 7](#)).
5. Wash dish 3 times with 1 mL of PBS.
6. Mix prewarmed 7.5 µl AlexaFluor 568-labeled gelatin with 192.5 µl 0.1% gelatin per MatTek dish thoroughly at room temperature. Do not generate bubbles. The mixture is good for up to 1 h (*see* [Note 8](#)).
7. Add 200 µl of the AlexaFluor 568/gelatin mixture onto the MatTek dish and incubate exactly 10 min at room temperature (*see* [Note 9](#)).
8. Wash 3 times with 1× PBS.
9. Incubate the dish with protein-free IMEM media for 15 min, *see* [Note 10](#).
10. Wash 3 times with 1× PBS.

11. Incubate the MatTek dish in 70% ethanol for 20 min or overnight.
12. Wash with and store in IMEM medium with antibiotics in the dark (e.g., wrapped with aluminum foil) and store at 4°C for up to several weeks.

3.4. Seeding Cells on MatTek Dishes Coated with AlexaFluor 568-Labeled Gelatin

1. BT549 cells expressing PKD1-eGFP or Src c-Src(Y527F)-eGFP are cultured in a T25 flask.
2. Aspirate the culture media and wash the cells with 5 mL 1× PBS.
3. Aspirate the PBS and incubate cell with 1 mL trypsin solution for 1 min.
4. Tap the side of the flask to shake off the cells.
5. Add 5 mL IMEM complete media to neutralize trypsin.
6. Pipet the IMEM media containing the cells using a 5-mL pipette and draw the cells in and out 4–5 times to break apart cell clumps.
7. Count the cell density and seed 20,000 cells on AlexaFluor 568 gelatin-coated MatTek dish obtained in **Subheading 3.3**.
8. Incubate the cells on the MatTek dish in a humidified, 37°C, 5% CO₂ incubator or on the microscope stage in an environmental chamber with similar conditions for about 3 h and then start to observe with Olympus TIRF microscopy.

3.5. Simultaneous Monitoring of the Dynamic Membrane Localization of GFP-Tagged c-Src with TIRF and IRM Microscopy

A number of invadopodia-related proteins colocalize at invadopodia demonstrated by epifluorescence and confocal imaging ([11](#)). Interference Reflection Microscopy (IRM) is the imaging of interference patterns created by the thin (on the order of a fraction of the wavelength of light) spaces between thin films, such as the space between a coverslip and an apposed cell membrane ([19](#)). IRM is very useful for looking at a cell's adherence to a substrate and the results can be quantified by image analysis ([1](#), [19–23](#)). Here, we describe a detailed procedure to monitor cell adhesion by IRM imaging with standard epi-illumination, also see [Note 11](#). And, we show a method to directly visualize the dynamic membrane localization of GFP fused c-Src(Y527F) by a combination of TIRF and IRM microscopy.

3.5.1. Adjustment of the Laser Beam Angle for TIRF Imaging

Optimal TIRF imaging occurs within 100 nm beyond the cover glass. Therefore, it is extremely critical to find the best laser beam angle at the exact focused position of the objective to obtain a high-quality TIRF image. Here, we present a detailed protocol for using a 488-nm laser for obtaining an optimal TIRF image. To do so, follow three major steps listed and also see [Note 12](#):

1. Prefocus the sample (cells) prior to laser adjustment:

- (a) Seed GFP expressing cells on an uncoated MatTek dish in cell culture incubator for 1 h before imaging or the point at which satisfactory cell-substratum adherence occurs.
 - (b) Select the Plan Apo 60× OIL/ N.A. 1.45 objective lens and apply a sufficient amount of immersion oil.
 - (c) Mount and center the cell sample dish on the stage just over the objective lens.
 - (d) Using the application software, direct the light path to the binocular eye pieces, and select the best objective position, focus, centering the cell(s) of choice using transmitted light.
 - (e) Using the application software, apply epi-illumination with the 488 filter cube to observe and find the best focus for adherent and well spread cells on the cover glass.
2. Focus laser beam on the ceiling.
- (a) Return the cell sample to the incubator.
 - (b) Clean the oil from the top of the 60× objective lens with lens cleaning paper or cotton-tipped applicators.
 - (c) Using the application software, direct the light path to the camera mode and turn on the laser beam so that it projects onto the ceiling (*see Note 13*).
 - (d) Insert an Allen wrench into the hexagonal socket adjacent to the position where the optical fiber inserts into the microscope (arrow b” in **Fig. 1b**) so that the laser beam will be projected to the ceiling vertically.
 - (e) Adjust the position of the optical fiber by sliding it in and out to find best focal plane (most concentrated, symmetrically shaped dot of laser intensity projected onto the ceiling).
 - (f) Use the Allen wrench to retighten the screw which is located on the underside of **Fig. 1b** (arrow b”) to maintain the laser focal position.
3. Find the best TIRF angle.
- The clear difference between the TIRF and Epi-illumination images is that there is little cytosolic “noise” signal in the TIRF images (**Fig. 2**). To obtain a high-quality TIRF image:
- (a) Apply a sufficient amount of immersion oil and return the cell sample to the microscope over the objective lens, and then repeat the earlier steps to obtain accurate focus and centering of the cell of interest, as described in **Subheading 3.5.1**.
 - (b) Using the application software, turn off the epi-illumination and then open the AOTF-controlled 488 laser shutter, set the light path to the camera, and select the 488 filter cube for AOTF.

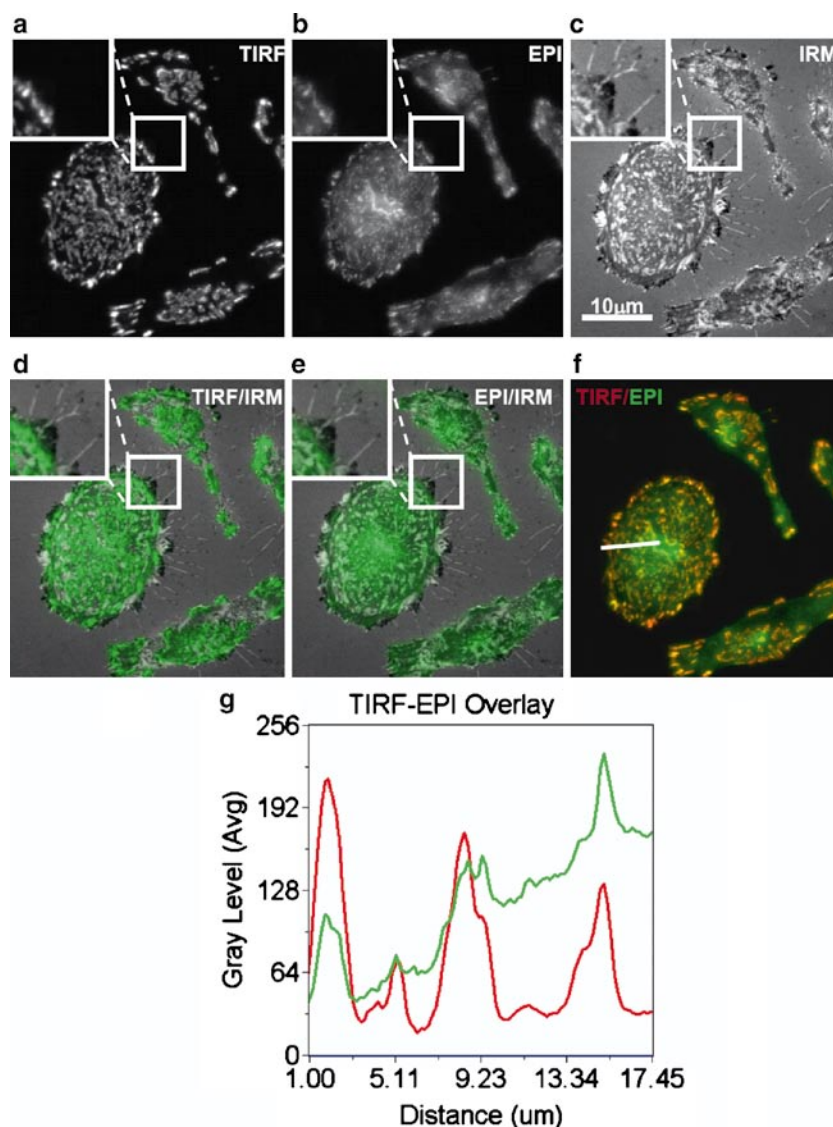


Fig. 2. Membrane localization of GFP-tagged Src in BT549 breast cancer cell line by TIRF and IRM microscopy. (a) TIRF image of the distinctive localization of Src(Y527F)-GFP in the adhesion sites. (b) Epi-illumination of the same cell (c) IRM image by standard epi-illumination by Olympus IX 81 with Plan Apo $\times 60$ OIL/ N.A. 1.45 TIRF objective with $\times 1.6$ optovar, Olympus. Scale bar indicates 10 μm . d, e, and f are overlay images of TIRF/IRM, Epi/IRM, and TIRF/Epi images, respectively. White line in f is for quantitative measurement of the intensity distribution of Src(Y527F)-GFP from the edge to the center of the cell in the overlay image of TIRF and Epi-illumination; this analysis (g) clearly shows a nearly uniform background in the TIRF image but an increasing background in the epi-illumination images.

- (c) Pull the Dual-View™ filter slide half-way out of the tube (called *Bypass Mode*) and the slider lever stays in *Band-Pass* position (see Note 14).
- (d) Using the application software, direct the light path to the camera and preview the image which at this point appears in epi-illumination mode (Fig. 2b). Adjust the screw con-

trolling the lateral movement of the laser in order to set laser beam angle for the best TIRF. This screw control is at the end of an extender and should be turned counter clockwise (**Fig. 1b**). As the incident angle is increased, the intensity of the signal decreases. At the correct TIRF angle, the intensity increases dramatically (**Fig. 2a**). Another sign that the best TIRF angle has been achieved is that if the screw control is turned even a small amount further, the cell signal will completely disappear. In that case, just turn the control level back (clockwise).

- (c) Adjustment of the TIRF angle will not be needed during a time lapse image acquisition. However, for each new experimental dish or even in different cells or fields of view, you will very likely need to adjust the beam angle refocusing the sample as you do so.

3.5.2. Adjustment of the Light Path for IRM Imaging with Standard Epi-illumination

Interference Reflection Microscopy (IRM) is a technique that requires well-centered epi-illumination and the precise control of several apertures in the light path to optimize image quality.

1. Center and balance the output of the Hg bulb in the fluorescence illumination light path.
2. For sequential imaging of TIRF using the laser and then IRM using the epi-illumination, use the Selection Prism (arrow b'' in **Fig. 1b**) at the junction of the split box at the rear port (beamsplitter) to allow ~20% transmittance from the epi-illumination and ~80% laser beam power to be routed to the sample (*see Note 15*).
3. Using the application software, direct the light path to the camera and select the 488 filter cube for wide field image acquisition.
4. Select the ND filter with the lowest transmission (1%) and preview to examine the quality/intensity of IRM imaging.
5. Adjust the size of the Aperture Stop (AS, a light intensity control after the Hg bulb and right before Selection Prism in the light path) so that light extends to just beyond the size of the preview window (**Fig. 1b** and see B', AS).
6. Adjust the Field Diaphragm or Field Stop (FS, a second light intensity control after AS before 20/80 prism in the light path) to obtain the best contrast of adhering membranes or structures and background (**Fig. 1b** and B', see FS).

3.5.3. Acquisition of c-Src Localization on the Plasma Membrane with TIRF and IRM Microscopy

For live cell TIRF and IRM imaging, it is critical to maintain the system accurately at 37°C (*see Note 16*). To set up a sequential IRM and TIRF imaging experiment:

1. Select the Plan Apo 60× OIL/ N.A. 1.45 objective lens and apply a sufficient amount of immersion oil.

2. Seed BT549 cells expressing c-Src(Y527F)-eGFP on an uncoated MatTek dish 1 hour prior to the imaging experiment so that the cells have a chance to attach.
3. Using the application software, direct the light path to the binocular eye piece and find the best objective position using transmitted light.
4. Using the application software, apply epi-illumination with the 488 filter cube to observe the cells, to further adjust and find the best focus of well spread cells on the cover glass and to choose a cell (**Fig. 2b**).
5. Using the application software, close the epi-illumination and direct the light path to the camera. Select the 488 filter with wide field image acquisition.
6. Pull the Dual-View™ filter slide half-way out of the tube (called *Bypass Mode*) and the slider lever stays in *Band-Pass* position.
7. Preview the images with the setting for TIRF imaging and check exposure time to obtain high-quality TIRF images (**Fig. 2a** and also see **Subheading 3.5.1**).
8. Preview with the settings for IRM imaging to obtain a high-quality IRM image and check exposure time to obtain best-quality IRM images (**Fig. 2c** and also see **Subheading 3.5.2**).
9. Set a sequential time lapse image acquisition (a single time point is illustrated in **Fig. 2**).

3.6. Simultaneous Monitoring of Protein Transportation and Invadopodia Formation with TIRF Microscope

1. Seed with BT549 cells expressing GFP-tagged PKD1 3 h before the experiment.
2. Select the Plan Apo 60× OIL/ N.A. 1.45 objective lens and apply sufficient amount of immersion oil (see **Note 16**).
3. Position an AlexaFluor 568-coated MatTek dish on the stage over the objective lens.
4. Direct the light path to the binocular eye pieces and find the best objective position using transmitted light.
5. Apply epi-illumination with the 488/568 filter cube to simultaneously observe GFP expressing cells with dark areas on the AlexaFluor 568 subtraction indicating an active formation of invadopodia (areas where fluorescent matrix is removed).
6. Close the epi-illumination and direct the light path to the camera.
7. Push the Dual-View™ filter slider into the tube (called *Dual-View Mode*) and the slider lever stays in *Dual-View* position.
8. Preview the images to obtain a high-quality, simultaneous, two-color TIRF image on the split screen.
9. Adjust the right exposure time to obtain high-quality dual view TIRF image (**Fig. 3c**, separated into single-color planes in 3A-B) (see **Subheading 3.5.1**).

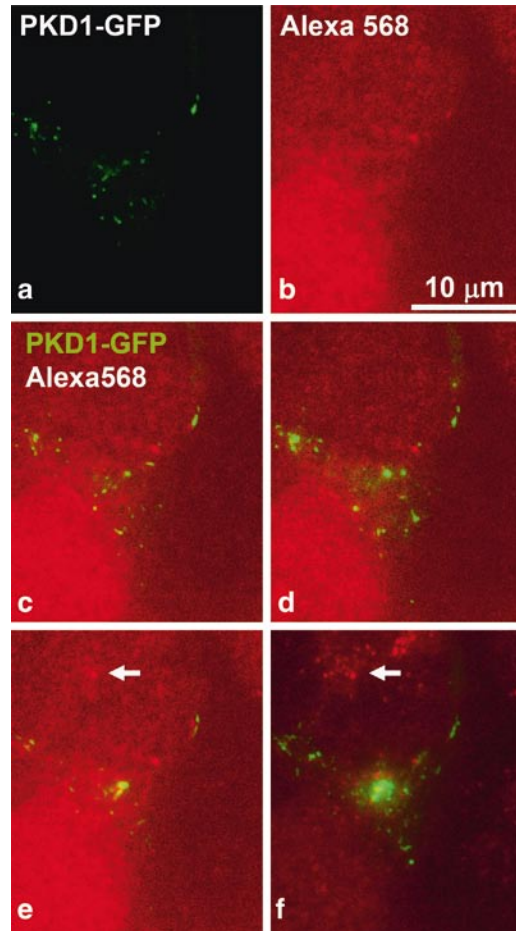


Fig. 3. Simultaneous monitoring of membrane transportation of GFP-tagged PKD1 and invadopodia formation by degradation of AlexaFluor 568-coated gelatin by Dual View TIRF imaging. A, B, and C are GFP, AlexaFluor 568 and merged images of Dual View imaging at the best laser focus for the GFP channel (A). D is a merge image for Dual View imaging at the best focus for Alexa 568 channel. In the image E, *arrow* points to a phagocytic particle of Alexa 568 gelatin which is also observed in image F. F. The same particle together with other phagocytic particles is visualized in the cell on the top left corner of the images by changing the TIRF beam angle. All the images were taken by Plan Apo $\times 60$ OIL/ N.A. 1.45 with $\times 1.6$ optovar, WD 0.1 mm with correction collar. Scale bar is 10 μm .

10. Set a sequential time lapse image acquisition with 1-s intervals.
11. Use MetaMorph Offline Ver. 7.5 to do further data analysis and presentation as shown in **Figs. 2–3**.
12. Turn the TIRF beam angle back (further clockwise) to increase the epi fluorescence image deeper into the cytoplasm. Intracellular AlexaFluor 568 particles can then often be observed which indicates that the degradation and appearance of dark spots in the ECM is at least partially due to the phagocytic process (**Fig. 3**).

4. Notes

1. Dissolve gelatin in a buffer free of ammonium ions or primary amines such as the PBS used in this chapter. The presence of a low concentration of sodium azide (≤ 3 mM) or thimerosal (≤ 1 mM) will not interfere with the conjugation reaction. Each vial of the reactive dye contains the appropriate amount of dye to label approximately 1 mg protein. 0.5 mL of a 2 mg/mL gelatin solution will be used for labeling (final concentration: 0.1%).
2. Achromat Doublets are used to correct chromatic aberrations for multiple excitation laser light directed through a single laser fiber. These lenses were mounted in the path of the TIRF straight illuminator (TIRF illuminator: IX2-RFAEVA-2-5). BL14026/101 is mounted after the exit of the laser light from the optical fiber and prior to the Field Stop. DBL14133/101 is mounted after the field stop and the total reflection mirror, and prior to the objective back focal plane. A diagram of the TIRF illuminator light path can be found in **Fig. 3** of ref. (18).
3. Gently insert the column through the X-cut in one of the provided foam holders. Using the foam holder, secure the column with a clamp to a ringstand. Carefully remove the cap from the bottom of the column. We often run three reactions simultaneously so that every step is done in triplicate.
4. Resin (Bio-Rad BioGel P-30 Fine Size purification resin) is designed to separate free dye from proteins with MW > 40,000. Make sure the buffer elutes through the column with a consistently even flow prior to adding the reaction mixture. If the flow of the buffer is slow or stalled, repack the column.
5. Be mindful that if the laboratory is below approximately 23°C, the gelatin solution will tend to gel and the column will not flow correctly.
6. 12.5 µg/mL poly-L-lysine is suitable to observe the invadopodia with TIRF microscope. 50 µg/mL poly-L-lysine is recommended for coating the coverslips for imaging the “holes” for confocal microscopy (10, 17).
7. Glutaraldehyde is a divalent crosslinker. It is used here to irreversibly crosslink gelatin to the glutaraldehyde pretreated polylysine coating. The gelatin itself is not crosslinked but binds to activated aldehyde groups associated with the polylysine after the glutaraldehyde is washed away. Dissolve 10 mL 8% stock Glutaraldehyde from a sealed ampoule plus 150 mL of PBS to make 0.5% Glutaraldehyde which will be suitable to use up to 1 month. Store at 4°C.

8. The ratio of AlexaFluor 568 and 0.1% gelatin is optimized for observation with the TIRF microscope. A mixture of 30 μ l AlexaFluor gel and 50 μ l 0.1% Gelatin per coverslip is recommended for confocal imaging by other researchers (10, 17).
9. All the procedures of coating AlexaFluor 568 gelatin on MatTek dishes are carried out in the tissue culture hood to maintain sterility. At this incubation step, it is recommended to turn off the fan and the light to decrease uneven evaporation and photobleaching.
10. A good use for outdated media, this step is to block free aldehydes with primary amines contained in the amino acids.
11. Dual-View™ provides a single TIRF image utilizing the entire camera field of view when the filter slider is in the half-way out position (called *Bypass Mode*) and the slider lever stays in *Band-Pass* position. Dual-View™ also provides a split double TIRF image when the filter slide is pushed all the way into the tube (called *Dual-View Mode*) and the slider lever stays in *Dual-View* position.
12. There are several manufacturers that make TIRF microscopes, and each product can use different software products to operate their systems. For the Olympus TIRF microscope, it is recommended by the maker to apply several options to operate the systems: MetaMorph, IPLab, and Slidebook. However, IPLab cannot control the Olympus ZDC automatic focus, whereas the other two can. Although there are differences in the details of how to operate the TIRF systems using the different software products, there are common principles/steps used to obtain high-quality TIRF images as described in this chapter.
13. Be aware of the safety device and adjust it so that the laser output to the sample is not blocked. This will allow the laser, in the absence of the sample and with the upper portion of the microscope pushed back out of the way, to strike the ceiling.
14. IRM can be performed by either epi-illumination or confocal laser imaging. In this chapter, IRM imaging is performed by standard epi-illumination due to the need for simultaneous TIRF imaging which requires a total internal reflection of the laser beam. A proper alignment of the epi-illumination is critical for IRM imaging.
15. Instability of the objective lens caused by thermofluctuation is one of the key factors affecting the quality of the TIRF image. For long-term live TIRF imaging of mammalian cells, the temperature chamber or the objective heater, if possible, should be maintained evenly and constantly at 37°C. However, it is possible to warm the objective to 37°C

at least 30 min before you do TIRF imaging experiments so that when the imaging starts, you have minimized thermal movements on the microscope.

16. We found it is difficult to obtain a good focal plan in which both GFP and AlexaFluor 568 were imaged with equal quality using the 150 $\times$ objective lens. Instead, we found it superior to use the 60 $\times$ objective lens along with the 1.6 $\times$ Optovar (mounted on the right side of the microscope body). This is almost equivalent to directly using a 100 $\times$ objective lenses. Olympus TIRF instruments outfitted with a multiport illuminator providing for separate focal alignment of individual laser lines would alleviate this issue. We have found that the insertion of doublet achromat lenses in the laser pathway and the use of the 60 $\times$ lens provide excellent quality of images with minimal laser attenuation.

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Chapter 15

In Vivo Assay for Tumor Cell Invasion

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Summary

We describe an in vivo invasion assay that enables the collection of invasive cells from the primary tumor. In addition to determination of the endogenous, unstimulated invasive properties of cells in vivo, the assay can take advantage of the chemotactic properties of cancer cells. Microneedles are filled with a mixture of extracellular matrix components such as Matrigel with or without a chemoattractant such as EGF, and then introduced into the primary tumor of a rat or mouse that is generated either by orthotopic injection of carcinoma cell lines or by a transgene such as polyoma Middle T. Over the course of 4 h the invasive cell population enters the needles while the animal is kept under anesthesia. At the end of the collection time, the invasive cells are extruded from the microneedles and can be analyzed in terms of the number and type of cells that invade in response to defined stimuli. By including pharmacological inhibitors in the needle, signaling pathways contributing to in vivo invasion can also be identified. This assay leads to a better understanding of the cell types and signaling involved in the tumor microenvironment, and has the potential to be applied to a variety of in vivo models.

Key words: Invasion, Chemotaxis, Tumor microenvironment, In vivo, Chemoattractant

1. Introduction

An important characteristic of cancer cells is their ability to detect chemoattractant gradients and move in response to them (1, 2). It has been shown that chemotaxis plays a critical role in metastasis, where cancer cells detect chemoattractant gradients found within the tumor microenvironment (3, 4) and distant tissues (5), and respond to these signals by leaving the primary tumor, entering the circulation and colonizing other organs (6, 7). In vitro studies of chemotaxis using devices such as the Boyden

microchemotaxis chamber allow for accurate measurements under controlled conditions, but often are restricted to studying cell motility on flat, 2-D surfaces. Increasingly sophisticated 3-D invasion assays help to provide more realistic, accessible models to study cell motility and invasion (8, 9), but still remain limited approximations that require comparison to cell behavior in a true *in vivo* microenvironment. To most accurately study the *in vivo* process of invasion at the cellular level, the direct collection of tumor cells *in vivo* was central to designing what we term the *in vivo* invasion assay (10). This assay enables the direct stimulation and collection of cells that have been conditioned and grown in their natural microenvironments. With this assay, such cells are directly collected from living animals. The assay can be used for a variety of purposes, including (1) measuring invasion under various conditions or stimuli, (2) evaluation of invasive cell types, (3) pharmacological dissection of invasion mechanisms *in vivo*, and (4) further characterization of the specific properties of the invasive cells. In the remainder of this section we provide some examples of these uses.

Measurements from the *in vivo* invasion assay have been shown to correlate with the metastatic properties of cancer cells in a xenograft model of breast cancer, with more metastatic cells invading 15-fold more efficiently than less metastatic cancer cells in response to an EGF gradient, correlating with their *in vitro* chemotactic responses to EGF (10). Transgenic models of breast cancer have also been examined, and the invasiveness of breast cancer cells to chemoattractants such as TGF- α and heregulin among others was evaluated in the polyoma Middle T model (11).

Identification of the invasive cells by immunofluorescence led to a remarkable and unexpected result. Both cancer cells and macrophages invaded into the needles, leading to the discovery of a paracrine communication between these cell types during invasion. Chemoattractant-stimulated secretion of CSF-1 by cancer cells can activate macrophages to secrete EGF, resulting in further stimulation of the cancer cells. This positive feedback loop can result in the propagation of the chemotactic signal (11). Inhibitors included in the needles with the chemoattractants that blocked either EGF or CSF-1 signaling lent further support to this model of paracrine signaling between tumor cells and macrophages (11). In addition to analyzing the invasive properties, further characterization of cell–cell and cell–stroma interactions in those tumors was achieved by intravital multiphoton imaging of the tumors during the invasion assay (11).

The collected invasive cells can be extruded from the needles and then characterized using other assays. Carcinoma cells from such *in vivo* invasion assays were subjected to cDNA microarray analysis and compared to the average primary tumor population (12,

13). Gene expression characterization of the invasive cancer cells collected using this assay has shown that invasive cancer cells activate motility pathways that are important for cells to leave the primary tumor, and are nonapoptotic and nonproliferative (12, 14). This method provides a way of overcoming the problem of identifying the gene expression signatures or other properties of a relatively small number of invasive cells within a primary tumor.

In summary, the in vivo invasion assay has provided us with vital information about how in vitro chemotactic properties of breast cancer cells correlate with their in vivo invasive and metastatic characteristics. The assay provides the conditions to generate a gene signature specific to the invasive population, as opposed to the average primary tumor. It was critical for the discovery of a paracrine interaction between breast carcinoma cells and macrophages which is important for their invasive properties. It is therefore evident that this in vivo invasion assay has many potential applications, such as testing effects of different chemoattractants, inhibitors, or ECM composition on invasion. This assay is not limited to studying breast cancer, and can be applied to a wide range of tumor models, whether created by cell lines injected orthotopically into different sites or tumors generated by different transgenes. This assay can potentially be applied to any tissue that has a migratory cell population.

2. Materials

2.1. Instruments for Needle Cell Collection Setup

1. *Micromanipulator.* We use the Narishige model MN-151 as a relatively inexpensive but stable micromanipulator.
2. *Needle holder.* We use a customized holder for the needles (Fig. 1). A 4 cm × 5 mm stainless steel bar is turned on a lathe so that the first disc is shaped to be 1 mm in width × 4 mm in diameter. The next part of the bar is shaped to be a 3 mm × 3 mm spacer, followed by another 1 mm × 4 mm disc, followed by a 5 mm × 3 mm spacer. Three equidistant 0.5 mm holes are drilled into each of the discs to hold the 25-gauge guiding needles. The bar is attached to a 12.5 mm × 1 mm disc with three equidistant 1-mm holes. It is silver soldered to a 12 cm × 4 mm stainless steel bar that is threaded at the far end. A Plexiglas block of 1 cm × 1.5 cm × 0.5 cm is tapped to take the threaded end of the bar. The block allows the holder to be placed in a Narishige model MN-151 micromanipulator.
3. Hamilton needles, 33 gauge (Fisher Scientific, Pittsburgh, PA).

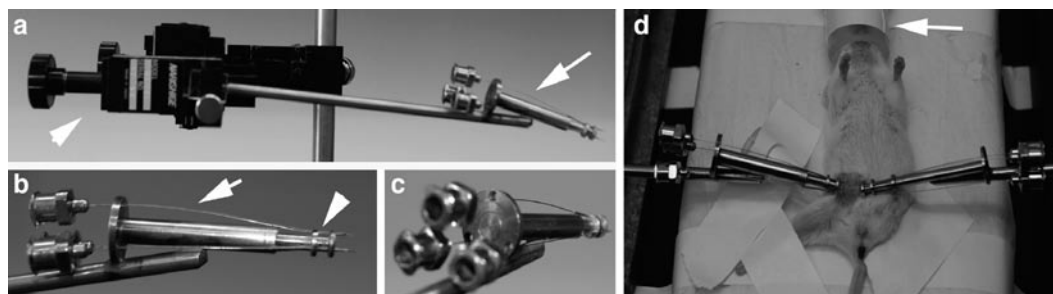


Fig. 1. In vivo invasion instruments and setup. (a) Apparatus used for the in vivo invasion assay. *Arrow* points to microneedle holder. *Arrowhead* points to Narishige model MN-151 micromanipulator. (b) Closeup of microneedle holder. *Arrow* points to 33-gauge microneedles. *Arrowhead* points to 25-gauge guide needles. (c) Rear view shows how microneedles are placed within the holder. (d) Experimental setup of microneedles inserted into an orthotopically grown mammary tumor in an SCID mouse. *Arrow* points to nose piece for delivery of Isoflurane anesthesia.

4. *Guide needles.* The guide needles are made by cutting standard 25-gauge needles at the plastic base. Prior to cutting, a wire is inserted into the needle to avoid closing the hollow part of the needle.
5. Vaporizer for Isoflurane, model 100 (Surgivet, Waukesha, Wisconsin).
6. Mapleson E. breathing circuit (VetEquip, Pleasanton, CA).
7. Experimental platform for laying down the rodent during the procedure. Use the styrofoam holders of 15-mL or 50-mL conical tubes, wrapped in a bench disposable diaper (Kendall).

2.2. Animal Models Used for Cell Collection

1. Xenograft model of breast cancer: our laboratory uses the rat mammary carcinoma MTLn3 cells (10). This cell line is grown in α -MEM supplemented with 5% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin solution (Life Technologies). The tumor cells are grown to 70–85% confluence before being harvested. Cells are detached using PBS–EDTA and scraped using a rubber policeman. 5×10^5 cells are injected into the right fourth mammary fat pad from the head of 5-to-7-week-old female severe combined immunodeficient (SCID) mice (National Cancer Institute, Bethesda, MD). Tumors are allowed to grow for 4 weeks before cell collection when the tumor is about 2 cm in diameter (see Note 1).
2. *Transgenic model of breast cancer.* FVB mice transgenic for the polyoma virus middle T (PyVT or PyMT) oncogene under the mouse mammary tumor virus (MMTV) long terminal repeat 206 (LTR), generated as previously described (15), are used for in vivo invasion assays at 12–14 weeks of age when the tumors are about 2 cm in diameter (see Note 2).

2.3. Reagents for Cell Collection

1. *L15-0.3% BSA solution*. Leibovitz's L-15 Medium (1×), no phenol red, stored at 4°C protected from light (Invitrogen, Carlsbad, CA). Bovine serum albumin, Fraction V, ethanol precipitated, stored at 4°C (Fisher Scientific). Prepare L15-0.3% BSA solution fresh for every experiment by first preparing a 6.9% BSA solution in PBS, sterile filtered using 0.20-μm sterile syringe filter. Keep the L15-0.3% BSA solution on ice covered with aluminum foil.
2. Matrigel basement membrane (Becton Dickinson, Franklin Lakes, NJ). Store at -80°C in 200–500 μL aliquots. Thaw single aliquots overnight at 4°C when you are ready to use. Always keep on ice to prevent gel formation. Store the thawed Matrigel aliquot at 4°C. Stock concentration of 9.7 mg/mL, diluted 1:10 in the needles.
3. Ethylenediamine tetraacetic acid, pH 8.0 (EDTA, Invitrogen). Prepare 1 mM stock solution by diluting with double-distilled deionized water. Store at room temperature.
4. Epidermal growth factor (EGF, Gibco Life Technologies). Dilute in PBS to make 50-mM stock aliquots and store stock at -80°C. Thaw an aliquot on ice the day of the experiment and dilute as needed with L15-0.3% BSA. Once EGF has been diluted with L15-0.3% BSA it should not be refrozen; discard at the end of the day.
5. Isoflurane, USP (Baxter, Deerfield, IL).
6. Oxygen tanks (M-E) with regulator (TechAir, Connecticut).

2.4. Reagents for Cell Counting

1. 4',6-Diamidino-2-phenylindole, dilactate (DAPI, Sigma, St. Louis, MO). Prepare a 10 mg/mL stock solution in ddH₂O and a 0.5 μg/mL working solution in PBS. Store both at 4°C protected from light.
2. Microscope cover slips, 24 mm × 50 mm × 1.0 mm (Thermo Scientific, Waltham, MA).
3. Sterile disposable 1-mL syringes with Luer slip tip (Fisher Scientific).

2.5. Reagents for Cell Typing by Immunofluorescence

1. Formalin neutral-buffered phosphate 10% (BioWhittaker, Walkersville, MD).
2. Poly-L-lysine hydrobromide (Sigma). Prepare a 0.1 mg/mL stock solution in water. Store at -20°C.
3. MatTek dishes (MatTek, Ashland, MA). To prepare the MatTek dish for cell plating, clean the dish in a culture hood by incubating with 1 M HCl for 5 min, rinse extensively with PBS, and then add 95% EtOH solution and incubate for another 5 min. Rinse the dish with PBS.

4. *Tris-buffered saline, pH 7.4 (TBS)*. Mix 8 g NaCl, 0.2 g KCl, 10 mL 1 M Tris base in 1 L ddH₂O and adjust pH to 7.4 with HCl; store at room temp.
5. TBS-2% FBS solution: dilute fetal bovine serum (FBS) (Gemini, West Sacramento, CA) to 2% with TBS.
6. TBS-1% BSA solution, sterile filter solution using 0.20- μ m sterile syringe filter. Store at 4°C.
7. 0.05% Triton X-100 (Sigma-Aldrich) in TBS. Store at room temp.
8. Primary antibody mixture of rabbit pan-Cytokeratin antibody (Santa Cruz) (1:50 dilution) for carcinoma cells and rat anti-F4/80 (16) (1:25 dilution) for macrophages. Store the undiluted pan-Cytokeratin antibody at 4°C; never freeze. The undiluted F4/80 antibody should be stored at -20°C in several aliquots; prevent frequent freeze-thaws. Use appropriate (antirabbit for the cytokeratin, antirat for the anti-F4/80) secondary fluorescent antibodies, stored as indicated by manufacturer, protected from light.

3. Methods

3.1. Preparing Solutions for Needle Collection

1. 100 μ L total volume is prepared to fill each 33-gauge experimental needle. Thaw all reagents on ice and prepare mixtures on ice using sterile 1.5-mL Eppendorf tubes. Each needle should contain:
 - 0.01 mM final concentration of EDTA in 100 μ L total volume
 - Dilute EGF stock with L15-0.3% BSA to a concentration of 1 mM, add 2.5 μ L of this dilution for a final concentration per needle of 25 nM (the normal concentration we use)
 - L15-0.3% BSA to bring total volume to 100 μ L, including the Matrigel volume
 - 10 μ L Matrigel (added last)
2. Vortex reagents at low speed to ensure proper mixing, getting rid of any bubbles in the mixture by tapping on the tube, place on ice.

3.2. Preparing the Needle Collection Setup

1. Clean setup with 70% ethanol, allow to dry.
2. Insert 25-gauge guide needles into the needle holder.
3. Place 33-gauge blocking needles through the 25-gauge guide needles, letting the tips of the blocking needles surpass the ends of the guide needles by several millimeters.

4. Place the needle holder into the micromanipulator.
5. Clean the 33-gauge experimental needles using a 1-mL syringe containing 70% ethanol. Pressing the plunger push about 0.5 mL of 70% ethanol through each needle. Get rid of any traces of ethanol by repeating the procedure with dH₂O.
6. Using a 1-mL syringe collect the contents of the Eppendorf tube with the appropriate mixture of buffer, Matrigel, and chemoattractant (such as EGF) prepared in **step 3.1** and load the 33-gauge experimental needles slowly until 2–3 drops of liquid are expelled from the needle tip (*see* **Note 3**).

3.3. Performing the Assay

1. Anesthetize rodent by using 5% Isoflurane, and place it on its back on the experimental platform, with the nose piece attached to the Isoflurane vaporizer placed around the rodent's head to allow comfortable breathing and delivery of Isoflurane (*see* **Note 4**).
2. Prepare the area of the tumor where the needles will be placed by trimming the hair using scissors, so there is a clear view of the tumor.
3. Using the micromanipulator, slowly advance the guide needles onto the area where the cells will be collected until the guide needles pierce the skin of the tumor (*see* **Note 5**). Make sure the guide needles truly pierce the skin of the tumor and that the setup is being held very stably (*see* **Note 6**). Gently push the blocking needles into the tumor until they are about 2 mm in (*see* **Note 7**). Carefully remove the blocking needles one by one, while holding the tumor in place. Do this paying attention that the guide needles do not move out of place.
4. Insert the experimental needles through the guide needles one by one, gently pushing them into the tumor until you feel resistance. Do not push any further as to avoid getting a tumor biopsy (*see* **Note 8**). Make sure that you hold the tumor in place each time you insert an experimental needle by supporting the tumor between your thumb and index fingers, so that the whole setup does not shift.
5. After all the experimental needles are placed in the tumor, watch the animal's breathing frequently to ensure proper anesthesia. Lower the Isoflurane level gradually, especially during the first half hour. There is variation from animal to animal, but after the first half hour Isoflurane should be for the most part no higher than 2%, the usual being around 1% and below (*see* **Note 9**).
6. Monitor the animal for the duration of the needle collection (we typically collect for 4 h), adjusting the Isoflurane level as necessary (*see* **Note 9**). At the end of the collection, carefully remove each experimental needle holding the tumor in place

by supporting the tumor between your thumb and index fingers. Remove the entire setup from the tumor and euthanize the animal by cervical dislocation.

7. Eject the contents of each experimental needle using a 1-mL syringe filled with DAPI onto a microscope slide. The contents of each needle should be emptied with no more than about 100 μ L of DAPI. Count the cells from each needle using a fluorescence microscope, counting all cells present within the drop by DAPI stain and/or other stains if cells are fluorescently labeled.
8. Clean experimental needles using a 1-mL syringe filled with 70% ethanol. Flush about 500 μ L ethanol through to clean each needle.

3.4. Identification of the Invasive Population

To characterize the invasive population, all the experimental needles should be used in this procedure (usually 3–6, depending on size of tumor) using the same experimental conditions (e.g., needles with chemoattractant) so as to maximize the number of cells available for imaging at the end of the procedure, for each experiment. There is a great loss of cells during this procedure as there are several incubation steps with washes in between.

1. Prepare a MatTek dish for extrusion of the needle collected cells by first rinsing the glass center with 1 M HCL for 5–8 min. Following this, rinse the dish with PBS, aspirate it, and add 95% ethanol solution for 1–2 min. After aspirating the ethanol and rinsing well with PBS, coat the glass part of the dish by adding poly-L-lysine solution for 2 h. At the end, aspirate the poly-L-lysine and allow the dish to dry before using it.
2. After the experimental needles are removed from the tumors carefully at the end of the needle collection, empty the contents of the needles into the precoated MatTek dish using a 1-mL syringe filled with 10% formalin solution. Total volume should not exceed 200 μ L. This means that only 2 or 3 drops should be extruded from each needle. Allow the cells to fix for 1 h.
3. To block nonspecific binding, add an equal volume of TBS-2% FBS solution (such that the final concentration is 1% FBS) and leave the dish overnight at 4°C.
4. The next day, remove the blocking solution and rinse 3 times with TBS-1% BSA solution. Keep in mind that all the washes and incubation solutions must be applied and aspirated extremely gently, by adding solution to one side of the glass part and aspirating from the other, never actually making contact with the glass surface where the cells are attached.
5. Permeabilize the cells with 100–200 μ L of TBS-0.1% Triton X-100 for 5 min at room temperature.
6. Wash 3 times with TBS-1% BSA.

7. Incubate the collected cells in a mixture of primary antibodies. For our purposes, we usually incubate with an anti-F4-80 antibody (1:25) and anti-pan-Cytokeratin antibody (1:50) in TBS-1% BSA to stain for macrophages and carcinoma cells, respectively. The incubation is done for 1.5 h at room temperature.
8. Wash 3 times with TBS-1% BSA.
9. Incubate in a mixture of appropriate secondary antibodies coupled to different fluorophores at a ratio of 1:300 (*see Note 10*) for 1 h.
10. Wash twice with TBS-1% BSA, and leave in the third wash. When ready, add DAPI and count the cells using a fluorescence microscope.

4. Notes

1. In our experience, the MTLn3 xenograft model is ready to be used for needle collection 4 weeks post injection in the mammary fat pad. At this point, the tumors are 1.5–2 cm in diameter and are not significantly ulcerated thus maximizing the surface area where the needles can be inserted. When using other xenograft models for this assay, one has to take into account the size of the tumor, which should be no smaller than 1 cm in diameter and not too ulcerated to allow the insertion of at least three needles.
2. When using transgenic models of cancer one has to first stage the tumor. For this assay we use tumors that are already invasive to maximize the number of cells that invade the needles. One can also compare the invasive potential of the cancer cells across different stages, but always making sure to use tumors at least 1-cm diameter to allow the insertion of at least three needles. Staging of the tumor can be confirmed after the experiment using H&E sections.
3. To determine the optimal concentration for collecting the invasive population, it is often necessary to perform dose response experiments and try a range of chemoattractant amounts. We have found that for EGF, the optimal concentration in the in vivo invasion assay is 25 nM while for the in vitro Boyden chamber chemotaxis it is 5 nM. The difference can be explained by different diffusion properties of the gradient emanating from a tip of a needle into live tissue vs. EGF in a buffer being directly accessible to migrating cells across pores in a filter. By the same token, the optimal

length of the invasion assay should be determined. Our group has determined that with EGF in the needles, the maximum number of cells was collected by 4 h (increasing in a linear fashion from time 0 to 4 h), after which the number of cells plateaus.

4. The position of the rodent during the experiment can and should be adjusted depending on the size and the position of the tumor. We commonly use orthotopic tumors in an abdominal mammary fatpad (2nd up from the tail) and place the rodent on its back during the experiment. In a different mammary tumor model such as the MMTV-driven Polyoma Middle T transgenic model, the mouse usually develops large, bulky tumors in the thoracic mammary glands; in such cases the tumor burden may affect proper breathing of the rodent, and we can overcome that by placing the animal on its stomach or on its side (with the largest tumor laying down on the surface), while the tumors remain easily accessible for the insertion of the needles. This trick may also be useful in cases when the animal is breathing heavily under anesthesia – to avoid the tumor shifting back and forth against the needles, one can stabilize it against the surface and additionally tape the tumor down. If the tumor is right in the middle of the rodent's ribcage and is weighing down on it, one can use two needle holders from each side, inserted in such a way as to almost be holding the tumor up.
5. Tumors may have different characteristics, but one should always palpate the tumor and try to select an area that is solid, and avoid the very soft necrotic areas which are unlikely to have viable cells that would be able to invade into the needle, and may also leak necrotic fluid out of the tumor.
6. Any wobbling or movement of the holder with the needles can later result in a failed experiment (for examples biopsy or empty needles).
7. One of the trickiest parts of the procedure is making sure the experimental needles are far enough into the tumor without taking a biopsy of it. How far they are pushed in can depend on how solid or soft the tumor mass is. As a first step, one should always make sure the guide needles are pushed enough against the tumor to have pierced the skin, and following this that the blocking needles are inserted far enough beyond where the tips of the guide needles seem to be. Sometimes forceps should be used to make sure the blocking needles are pushing through the tumor tissue, remembering that this should *not* be done with the experimental

needles. To increase the likelihood of experimental needles being inserted correctly, it is best to use more than one holder with multiple needles in each if the tumor size permits it. Some tumor types are too small to allow more than one holder to be used.

8. Occasionally the needles pierce a blood vessel or a necrotic area inside the tumor; this is unavoidable at the time of setup. If most of the experimental needles are already set up, it is best to not try and reinsert the experimental needle that seems to have been placed unsuccessfully in order to avoid disrupting the entire setup. Depending on the severity of the bleeding, the needle may or may not produce useable data. One should observe whether the bleeding is severe enough to cause premature death of the rodent during the experiment.
9. To avoid the animal dying from an overdose or waking up from insufficient levels of anesthesia, one must constantly observe the animal and adjust the levels of Isoflurane accordingly. As the rodent's breathing slows down (1 breath roughly every 3–5 s, but this is extremely variable from animal to animal), you can start lowering the Isoflurane in 0.5% steps. Constantly monitor the breathing and keep lowering during the first half hour as appropriate. If the rodent starts breathing faster at any point during lowering you should increase the Isoflurane as needed until the animal's breathing slows down again. The way the anesthesia levels are adjusted is rarely identical from one rodent to the next; variations can be due to the rodent's general health condition, its size and age, and also on the setup itself. One must verify that the oxygen tank has sufficient levels of gas and that the vaporizer has an appropriate amount of Isoflurane. It is helpful to watch for too frequent or too infrequent breaths of the animal. It may be helpful to place heating pads under the animal to prolong its survival as well.
10. Adjust ratio as needed for different antibodies.

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Chapter 16

Quantitative Studies of Neuronal Chemotaxis in 3D

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Summary

During development a variety of cell types are guided by molecular concentration gradients to form tissues and organ systems. In the nervous system, the migration and neuronal pathfinding that occurs during development is organized and driven by “guidance cues.” Some of these cues are substrate bound or nondiffusible, while many are diffusible and form gradients within the developing embryo to guide neurons and neurites to their appropriate destination. There have been many approaches used to discover and characterize the multitude of guidance cues, their cognate receptors, and how these cues and receptors are regulated to achieve the highly detailed connections found in the nervous system.

Here we present a method for creating precisely controlled gradients of molecular factors within a three-dimensional culture environment. The method is based on a non contact mediated delivery of biomolecules to the surface of a collagen gel. The factors are printed in a pattern on the top of a gel containing the tissue or cell type of interest embedded in the gel. The formation of the gradient is dependent upon the diffusion of the printed molecule in the gel. The concentration of the factor within the gel becomes independent of depth rapidly, and the gradient becomes smooth on a similar time scale. The gradients formed can remain relatively stable for a day or more. Moreover, the steepness and molar concentration of tropic or trophic factors within the gradient can be controlled.

Key words: Chemotaxis, Molecular gradients, Collagen gel, Axon guidance, Diffusible factors, Guidance cues, Cell motility

1. Introduction

Understanding how the nervous system is wired up during development is important for understanding both how neurological disorders based on wiring defects can be avoided, and how axons can be made to regenerate appropriately after injury. In the past 15 years or so several families of guidance molecules

have been shown to be important for guiding axons *in vivo*, including the Netrins, Semaphorins, Slits, Ephrins, Wnts, and some morphogens such as Shh (1–4). Much of our current knowledge about the mechanisms by which such cues guide axons comes from a small number of *in vitro* assays (2). The most influential of these are the collagen gel coculture assay (5) and the “pipette” or “growth cone turning” assay (6). In the former, neurons are embedded in a three-dimensional collagen gel close (a few hundred microns) to a source of the putative chemotropic factor, such as a tissue explant or a block of transfected cells. The chemotropic factor diffuses from the source to create a gradient in the gel, and the trajectories of axons emerging from the neurons are examined after 1–2 days in this environment. This assay recapitulates the timescale and 3D environment of many axon guidance events *in vivo*, but does not offer any knowledge, control, or stability of the gradient steepness and absolute concentration (7). In the pipette assay the chemotropic factor is expelled into a fluid environment from a narrow pipette about 100 μm from the tip of an individual axon growing on the surface of a culture dish. Providing convection currents are carefully controlled a relatively stable gradient can be established by diffusion from the pipette. The response of the axon to this gradient is then examined over timescales of about 1 h. Although this assay offers more control over gradient conditions than the collagen gel coculture assay, steepness and absolute concentration are still variable and hard to predict (8), and the timescale and dimensionality are less like the *in vivo* situation. Motivated by these limitations, we investigated whether it was possible to design a long-term collagen gel-type assay which would allow precise knowledge and control of the gradient conditions.

A variety of previous techniques have been developed for producing controlled gradients of chemical factors in three-dimensional gels. A simple approach is to place the gel in contact with a chamber of solution or gel containing the factor of interest. Diffusion of the factor creates a continuously evolving gradient (9–11). A more controlled gradient can be produced by placing a gel between two chambers with differing concentrations of factor. After an initial transient, a linear gradient is established between the two chambers (12–15). The main drawbacks of this method are that the gradient is established only after an initial transient that can be fairly long for large proteins and other slowly diffusing molecules, the reservoirs require a large excess of chemical factor, only linear gradients can be produced, and a separate apparatus is required for each culture dish. Protein gradients have also been encapsulated in a microporous gel by creating a linear gradient in a mold before gelation (16). There are many means of generating gradients of guidance cues, permissive, and

nonpermissive substrates in two-dimensional cultures which will not be discussed here.

We have developed a technique for gradient generation that relies on a computer-controlled micropump to create patterns of chemical factors on the surface of a relatively thin gel (17,18). As detailed later, this approach offers several significant advantages over most existing methods: There is no contact between the micropump and the gel, gradients can easily be reproduced in multiple experimental chambers, a variety of gradient shapes can be generated with the same hardware, no excess factor is required, and the gradients are established quickly. The shape of the gradients evolves in time in a way that can be accurately modeled by the diffusion equation. This evolution is the main drawback of this method compared to those described above, which can produce nearly stable gradients after sufficient time. However, the slow diffusion of many large proteins results in gradients which are stable for a day or more, which is adequate time for many in vitro studies. This technique can be used to investigate the role of gradients of diffusible substances in processes such as chemotaxis, morphogenesis, and pattern formation, as well as for high-throughput screening of system responses to a wide range of chemical concentrations. Moreover, multiple gradients of arbitrary spatial orientation can be applied, to examine the interaction and effect of a number of different biomolecules.

2. Materials

2.1. Tissue Preparation

1. P2 Sprague-Dawley Rat pups.
2. Dulbecco Modified Eagle's Medium (DMEM), high glucose, no glutamine, no pyruvate (Cambrex).
3. Ca^{2+} – Mg^{2+} free Hanks Balanced Salt Solution (Cambrex).
4. 2.5% trypsin solution (Cambrex) diluted 1:10 in HBSS for 0.25% final, aliquot and store at -20°C , stable for 1 year.
5. DNase I (Boehringer Mannheim) prepare 1 mg/mL stock in PBS aliquot store at -20°C stable for 1 year.
6. Heat-inactivated FBS (Cambrex) aliquot and store at -20°C .

2.2. Collagen Preparation

1. Tissue-culture-grade water (Cambrex).
2. Powder OptiMEM made to 10× OptiMEM (Invitrogen).
3. Tissue-culture-grade sterile 7.5% sodium bicarbonate solution (Sigma).

4. Penicillin/Streptomycin/Fungizone solution (100×) (Bio-Whittaker).
5. Type I rat tail collagen, 3–4 mg/ml solution (Becton-Dickinson).

2.3. Tissue Embedding in Collagen Gel

1. Dissection microscope (Nikon).
2. 35-mm tissue culture dishes (Corning).
3. Custom acrylic dish holder.
4. Printed gradient template.

2.4. Gradient Printing

1. Pump head microdispenser (Gesim #A010–001).
2. Multidose pump head controller (Gesim).
3. x-y translational stage (Velmex Inc., Bloomfield, NY).
4. Labview software (National Instruments, Austin, TX).
5. Custom-machined culture dish holder.
6. High-resolution video camera with zoom.
7. Video camera monitor.
8. Murine 2.5S NGF (Roche Diagnostics).
9. Capillary tubing 1 mm inner diameter with a luer lock end (Fisher).
10. 10-cc luer lock syringes (Becton-Dickinson).
11. Nanoplotter 2.0 (Gesim) (Optional, replaces items 1–7).
12. Standard 5% CO₂ tissue culture incubator with high humidity.

3. Methods

3.1. Tissue Preparation

Follow protocols for tissue preparation appropriate for culturing in collagen. Protocols for rat DRG harvesting and digestion have previously been described ([17](#)) (*see* [Note 1](#)).

1. Remove the DRGs from the lumbar region of P2 rat pups and place them in DMEM on ice.
2. Trim the DRGs of any nerve bundles or extraneous material.
3. Enzymatically digest the DRGs in 0.25% trypsin/10 µg/mL DNaseI/Ca²⁺- and Mg²⁺-free HBSS for 12 min.
4. Stop the digest with addition of an equal volume of heat-inactivated FBS.
5. Pellet and resuspend the DRGs 3 times in DMEM, then place on ice.

3.2. Collagen Gel Preparation

1. Prepare 10× OptiMEM from powder according to the manufacturer's instructions except use one-tenth of the specified final volume (*see Note 2*).
2. Based on the concentration and volume of type I rat tail collagen stock to be used, calculate the volumes of 10× OptiMEM, 100× Pen./Strep./Fung., 7.5% sodium bicarbonate solution, and water needed to formulate collagen gel solution (2 mg/mL collagen, 1× OptiMEM, 1× Pen./Strep./Fung.), factoring in that 27 µL of 7.5% sodium bicarbonate must be included for every mL of collagen stock used.
3. Prepare the collagen gel solution on ice (*see Note 3*), adding its components in order as follows (1) water, (2) 10× OptiMEM, (3) 7.5% sodium bicarbonate solution, 100× Pen./Strep./Fung., then the collagen stock, making the solution homogenous by pipetting up and down.
4. Using a 1,000-µL micropipettor, pipette 0.75 mL of the collagen solution into the bottom of the 35-mm culture dishes. Swirl and tilt the dish to make sure there is even coverage. The resulting "bottom collagen" gel will be about 0.5 mm thick. Plate the bottom collagen for all required experimental dishes and allow to gel for ~20 min at 21°C or in the tissue culture incubator.

3.3. Tissue Embedding in the Collagen Gel

1. Make a custom acrylic dish holder that snugly fits the culture dishes. The same brand and exact tissue culture dishes are used for all experiments. The bottom of the acrylic dish holder should be no more than 4 mm thick, and the holder must be shallow enough to allow the edges of the dish to be handled.
2. Create a template as shown in **Fig. 1** in a graphics program. In this instance the circular area is exactly the size of the bottom of the 35-mm dishes. The various lines of the gradient to be printed as well as the pregradient are denoted and are 1 mm apart.
3. Tape a printout of this template to the bottom of the acrylic dish holder. The printing template is used to ensure consistent explant placement and gradient production.
4. Wash the explants to be embedded in cold collagen solution. First remove the majority of the DMEM from the explant containing dishes leaving just enough to keep the explants moist. Place a second 35-mm culture dish on ice and pipette 250 µL of collagen solution into the dish. Using forceps transfer six explants into the collagen solution while trying to minimize transfer of DMEM.
5. Embed the explants in the collagen gel. Pipette 0.75 mL of collagen solution (this will be the "top collagen") into one of the dishes containing the already gelled "bottom collagen."

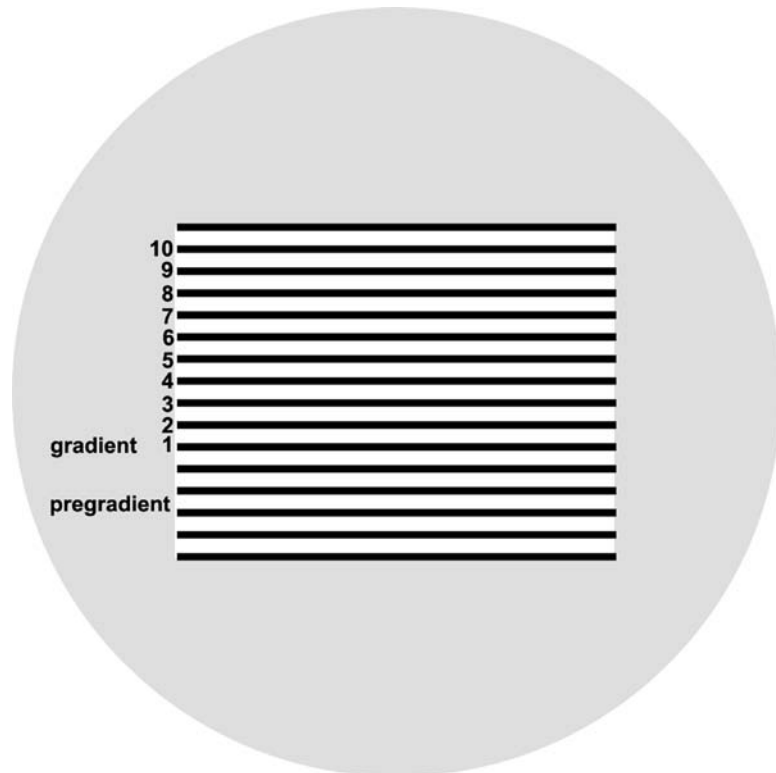


Fig. 1. A template created in a graphics software application to be used for embedding tissue in the collagen gel. This same template can be used to ascertain that the lines or spots of the gradient array are printed at the proper location within the culture dish. The template should be sized to exactly match the printed pattern.

Transfer the six explants into the top collagen and place the dish in the acrylic holder with the gradient template on the stage of the dissection microscope.

6. Position the explants in the appropriate coordinates within the collagen gel. Using forceps gently position the explants within the gradient. Mark the culture dish with a permanent marker to denote the gradient orientation. For the 10 line gradients used in (17), explants are placed between the third and fourth lines of the gradient, the region that is most stable in steepness and molar concentration (*see Note 4*). The explants should be placed such that they are evenly spaced, well separated, and not within 2 mm of the sides of the gradient (*see Note 5*). A gradient that is 20 mm wide can easily accommodate 6 DRG explants in this manner (*see Note 6*).
7. Allow the top collagen containing the explants to fully gel (usually 5–10 min) before moving the culture dish to the tissue culture incubator. Failure to allow collagen to fully gel

will result in disturbing the collagen matrix when the plate is moved, which can cause improper placement of the explants and spurious neurite guidance through the areas of disturbed collagen matrix.

8. Continue embedding the explants in this manner, changing the collagen solution in which the explants are washed every three dishes prepared (*see* **Note 7**).

3.4. Gradient Printing

1. Choose one of the following approaches to providing the required increase in guidance cue from one line to the next:

Approach a) Different stock solutions can be loaded into the pump head for each line printed while keeping the amount of solution printed constant from line to line. This method has the advantage that a constant amount of solution is printed, so a countergradient of vehicle is not required. However the pump head must be loaded a number of times, which requires washing and drying between each loading of a new solution. This approach is most suited to gradient production using the Gesim Nanoplotter (*see* alternative method later).

Approach b) The amount of guidance cue solution printed can be varied from line to line. The advantage of this approach is that typically only two solutions need be loaded into the pump head: the guidance cue and the vehicle for printing the countergradient and pregradient, as discussed later.

2. Adjust the microdispenser parameters to ensure droplet ejection. There are a number of settings, such as the voltage, pulse duration, and pulse frequency that affect the output of the microdispenser. The values of each of these settings must be tailored to ensure proper droplet ejection. A stroboscope is available from Gesim that enables visualization of ejected droplets, and the fine tuning of these settings to ensure a reliable droplet ejection.
3. Calibrate the microdispenser output. Each pump head is individually manufactured and calibration data are provided with each pump. The typical output for one pulse is on the order of 1 nl, depending on the voltage and pulse duration. We have found that with a pulse duration of 100 μ s and a voltage of 60 V most pump heads provide a droplet size of 1.4 nl. The output can be measured by using a Metler balance to measure the change in mass of a microcentrifuge tube caused by injection of 1×10^6 water droplets from the pump. We have also found that the pump heads have a reliable output independent of viscosity, by measuring the output of a BSA PBS solution ranging over three orders of magnitude, from 0.001 to 1% BSA.
4. Calculate the number of droplets and concentration of stock solution in order to achieve the desired molar concentration of the guidance cue at the location of the explants. The gradients

are created by printing ten lines of solution each 1 mm apart onto the surface of the collagen gel. Gradients 20 mm wide accommodate 6 DRGs without the neurites from the separate DRGs encountering one another. Because the collagen gel is 1 mm thick and the lines are spaced 1 mm apart, there is 20 μL of gel per line. Therefore the molar quantity of guidance cue applied will diffuse into the 20 μL bed volume to result in the final concentration required at that point in the gradient. An example of the necessary calculation is displayed in **Table 1**.

5. Determine the change in factor from one line to the next to attain the desired gradient shape. To form a linear gradient the quantity is increased by a constant amount from one line to the next. For exponential gradients the quantity is increased by a constant ratio. For a concentration change of $x\%$ across 10 μm (the typical size of a axonal growth cone), the ratio is $(1 + x/100)^{(100)}$. (The factor of 100 is the spacing between lines divided by 10 μm .). See **Table 1**.

Table 1

The parameters required to create a 10-line exponential gradient of 0.2%, 1 nM NGF at the explant using incremental deposition of the volume of chemotropic factor onto the collagen gel, when the concentration of the stock remains constant in the microdispenser

Line #	Pump stock (nM)	Droplets	Final concentration (nM)	Countergradient droplets
1	117	82	0.67	491
2	117	100	0.82	473
3	117	122	1	451
4	117	148	1.22	424
5	117	181	1.48	391
6	117	221	1.81	351
7	117	270	2.21	303
8	117	329	2.70	243
9	117	402	3.29	171
10	117	490	4.01	82

The explants are placed between the third and fourth lines, the exponential factor = 1.22, droplet size = 1.4 nL, the final bed volume/line is 20 μL . The total aqueous volume applied including countergradient ~573 drops; therefore, a pregradient of 5 lines of 573 drops of PBS is applied

6. Calculate the number of droplets required to form a countergradient (in the case of approach b). Because the addition of different numbers of droplets in different gradient lines may change the local collagen concentration or have other more subtle symmetry breaking effects, we also apply a countergradient of vehicle (PBS) so that the number of drops printed on each line is the same. Specifically, the countergradient is chosen such that the number of gradient drops printed on each line plus the number of countergradient drops printed on each line is equal to the number of drops printed on the line with the largest number of drops.
7. Calculate the number of droplets required to form a pregradient (in the case of approach b). We have also found that application of a 5-line pregradient of vehicle containing the same number of droplets present in the gradient and countergradient helps to reduce artifacts. In particular, we have found that the gradient of collagen density that can be present when the tissue is close to the edge of the printed region can sometimes produce a weak guidance effect by itself, confounding the effect of the chemotropic factor gradient.
8. Confirm that the lines of the printed gradient will be in the desired coordinates of the culture dish. The gradient pump system is controlled by a custom Labview program (available upon request). The program consists of a subroutine that ejects droplets from the pump head at a specified rate, a routine that moves along each gradient line at a specified rate, a routine that moves between lines, and a shell that accepts user inputs and performs calculations to direct the subroutines. Apply a gradient template (**Fig. 1**) to the bottom of a culture dish, and place the culture dish in the x - y translation stage holder. Load the pump head with vehicle (see below for loading procedure). Print a gradient or line and adjust the initial x - y coordinates until the printed gradient matches the lines on the template.
9. Load the stock solution of guidance cue into the pump head. If the pump head is not clean and dry, wash and dry the pump head as described later. Use a short (~1 cm) length of the capillary tubing that has been attached to 200- μ L micropipettor tip the very end of which has been removed to allow easier flow, attached to a 20- or 200- μ L micropipettor (*see Note 8*). Take the solution up into the capillary tubing and gently attach the capillary tubing to the pump head inlet (**Figs. 2 and 3**). Tilt the micropipettor upward and allow the solution to flow into the pump by gravity. If the pump head does not fill by gravity, a small amount of pressure can be used by depressing

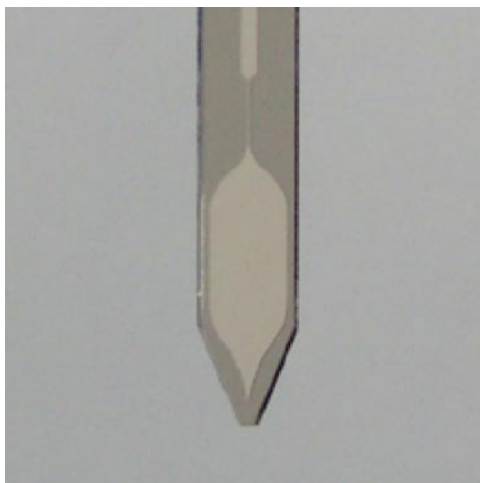


Fig. 2. A magnified view of the Gesim pump head (microdispenser), showing the piezo-activated reservoir.

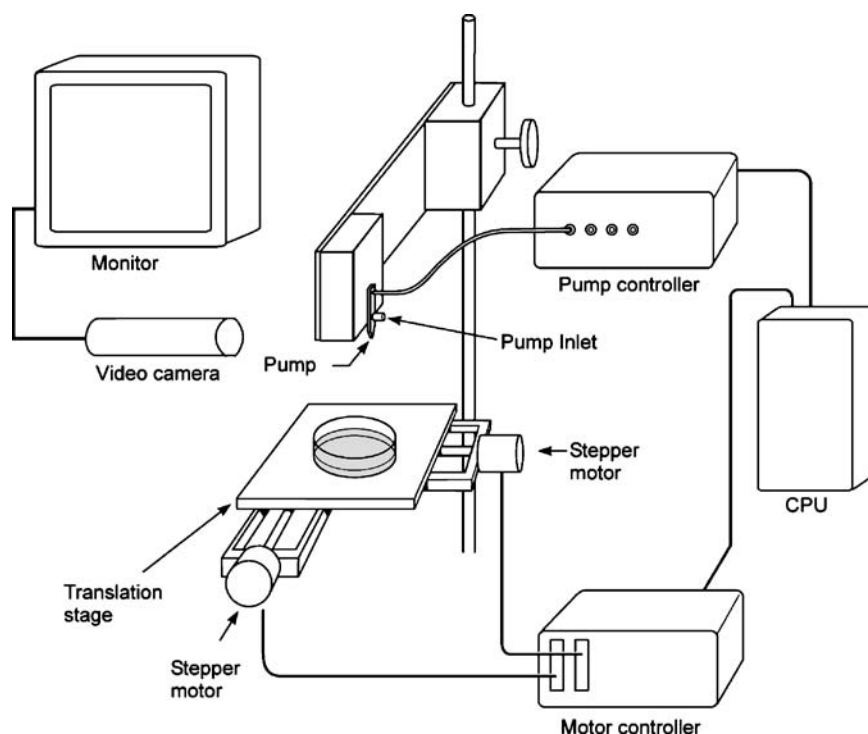


Fig. 3. A schematic representation of the original pump applied gradient generating apparatus. The CPU or computer runs the required program to control the microdispenser control unit, and the motorized translational stage. The video camera and monitor are used to ensure proper filling, washing, and drying of the microdispenser (17, 18).

the micropipettor actuator. Once the pump head is filled, gently eject the tip from the micropipettor.

10. Confirm that the pump head is properly loaded and ejecting droplets prior to printing a gradient. Examine the pump head for air bubbles on the video monitor (**Fig. 3**). If air bubbles are present, droplets will not eject properly, and the pump head must be washed and dried thoroughly (see later) and then reloaded with solution. Hold a piece of paper under the pump head while the dispenser is activated to check for droplet ejection.
11. Print the gradient (in the case of approach b), or individual lines of the gradient (in the case of approach a). While printing the gradient, or lines of the gradient, closely examine the surface of the collagen gel to ensure that droplets are properly deposited on the gel.
12. Wash the pump head. Take 2–3 mL of H₂O into a 10-cc luer lock syringe that has ~ 6 cm of capillary tubing attached to the luer lock. Attach the end of the capillary tubing to the pump head inlet and gently apply pressure to flow 2 mL of H₂O through the pump head. The tubing and the syringe are then removed and the pump head is dried externally with a Kimwipe.
13. Remove all remaining H₂O and dry the internal chamber of the pump head. Another such 10-cc syringe with capillary tubing is attached to pump inlet. Then a gentle vacuum is applied by pulling out the plunger of the syringe out. Repeat the application of the vacuum three times while examining the pump head on the video monitor **Fig. 3**. There should be no H₂O remaining. The microdispenser pump head must be washed and dried between each change of solution in the pump head, and after use for storage.
14. Print all remaining lines of the gradient (in the case of approach a). Reload the pump head with desired stock and print each successive line of the gradient. Wash and dry the pump head between loadings of each new stock solution, and application of each new line of the gradient.
15. Print the countergradient (in the case of approach b). Load the pump head with vehicle and print the countergradient as described earlier for the gradient. Wash and dry the pump head.
16. Print the pregradient. Load the pump head with vehicle and print the pregradient as described earlier for the gradient. Wash and dry the pump head.
17. Alternative method for approach a. An alternative method, which allows for greater automation of and control over some of the steps described earlier, is to use a device such as the

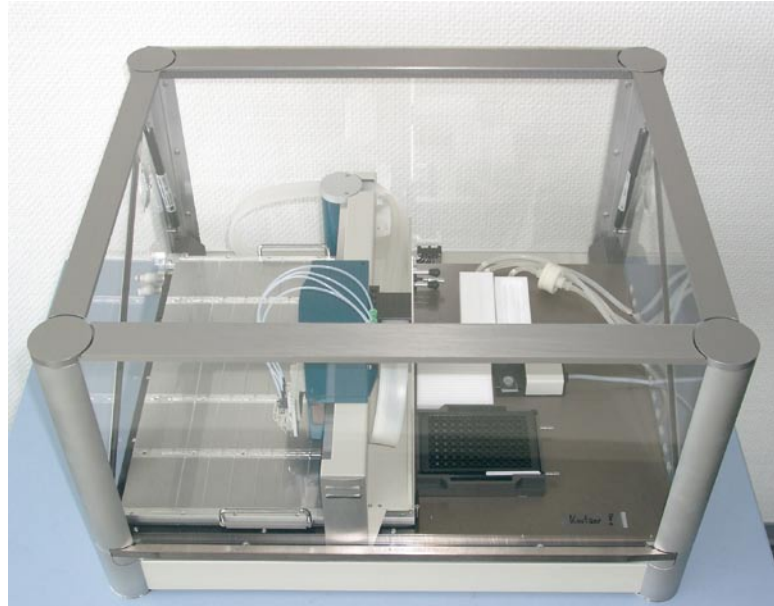


Fig. 4. The Gesim nanoplotter, a robotic fluid delivery system that facilitates the production of chemotropic gradients.

Gesim Nanoplotter (**Fig. 4**) (see www.gesim.de), which is a commercially available high-precision liquid delivery system. Although this device was originally designed to create proteomic and DNA arrays, it can be easily programmed to provide gradient patterns. The nanoplotter is robotic, and can contain a number of picoliter delivery piezo microdispensers, automated wash system, drying pad, and stroboscope with pattern recognition software. Chemotropic solution is taken up by the microdispensers from multiwell microtiter plates, and then is deposited on the collagen gel in an array. Instead of printing lines, in our current usage each line in the gradient is now made up of 20 spots 1 mm apart (*19*). Because the nanoplotter has an automated wash system to clean the microdispensers every time sample is taken up and printed, a constant volume of different concentrations of chemotropic factor can easily be placed in each line, and no countergradient is required. Moreover, the stroboscope and the pattern recognition software ensure that the microdispenser is ejecting droplets. If there is a failure in droplet ejection the nanoplotter will wash the microdispenser and repeat the sample pick up. If droplet ejection fails a second time, the nanoplotter notes this and moves on to the next sample while creating a program that can be ran after printing to correct for any errors in sample delivery. Steps for using the nanoplotter are as follows:

- a) Calculate the number of droplets and concentration of stock solution in order to achieve the desired steepness of the gradient and the molar concentration of the guidance cue at the location of the explants. An example of the stock solutions and calculations to create a gradient in this fashion is displayed in **Table 2**.
- b) Program the nanoplotter to deliver the required number of droplets from each well of the microtiter plate to the lines of the gradient. The nanoplotter has a number of transfer programs that will deposit solutions from the microtiter plate to the slide target area. Create the program as per the Gesim Nanoplotter manual.
- c) Confirm that the spots of the printed gradient will be in the desired coordinates of the culture dish. Apply a gradient template **Fig. 1** to the bottom of a culture

Table 2

The parameters required to create a 12-line exponential gradient of 0.2%, 1 nM NGF at the explant using a constant volume of chemotropic factor deposited onto then collagen gel, when varying the concentration of the stock in the microdispenser

Line #	Pump stock (nM)	Final concentration (nM)
1	110	0.55
2	134	0.67
3	164	0.82
4	200	1.0
5	243	1.22
6	297	1.48
7	362	1.81
8	442	2.21
9	539	2.70
10	657	3.29
11	802	4.01
12	978	4.89

The explants are placed at fourth line, the droplet volume is 0.5 nL for picodispenser. The final bed volume/line is 20 μ L. With 20 spots/line, 10 droplets/spot $\sim$ 0.1 μ L volume is applied/line. The exponential factor = 1.22. A pregradient of 4 lines, 20 spots/line, 10 droplets/spot of PBS is applied

dish, and place the culture dish on the desired location of the slide tray of the nanoplotter. Pipette vehicle into the wells of the microtiter plate and run the transfer program. Adjust the placement of the culture dish until the spots delivered by the nanoplotter coalign with the lines of the gradient template.

- d) Load the microtiter plate with the necessary stock solutions. Include the pregradient vehicle in the appropriate wells.
 - e) Run the nanoplotter program. If errors in microdispenser loading and ejection occur, “repair” programs will be generated. Run the repair programs as necessary.
18. Once the gradients have been printed, incubate the culture dishes containing the explants in a 37°C 5% CO₂ tissue culture incubator for 36–40 hs (*see Note 9*).

4. Notes

1. All efforts should be made to maintain the DRGs on ice to increase viability. Leibovitz L-15 medium can be substituted for the DMEM.
2. The 10X OptiMEM is the most critical component of the mixture. As the collagen gel will be moist with no extra liquid media overlaying the gel, the only initial source of nutrients will be the OptiMEM included in the collagen mixture. The final volume of OptiMEM should be made in a volumetric flask. All glassware and stir bars for media preparation are only used for this purpose and never exposed to detergents. A 10X concentration of other media can be substituted depending on the cell type or tissue used. Note this is a serum-free culture system.
3. Because collagen stocks vary in concentration, the volume of each component of the collagen gel solution will have to be calculated for each stock. Prepare Collagen solution in ice with all cold components, just prior to the DRG digestion. If air bubbles are introduced the solution can be centrifuged at 1,000 x g, 4° C for 5 minutes to remove the bubbles. The solution once made will eventually gel even if left on ice after about 3 hrs. Each 35-mm culture dish will require approximately 1.8 mL of collagen solution. The minimum collagen stock concentration that can be used to achieve a 0.2% gel is 2.32 mg/mL. A low concentration of NGF (0.1nM), N2 supplement (Gibco) or other neurotrophic factor can be

included in the collagen to help promote survival of the explants or cells. We have found this to not be necessary for DRGs if the gradient is applied within a few hours of embedding.

4. Larger gradients that contain more lines can be more stable. If a gradient with more lines is desired adjust the placement of the explants to be $\sim 1/3$ up the gradient from the bottom of the gradient.
5. We have found through finite element analysis that the concentration of guidance cue likely diminishes within 2 mm of the sides of the gradient.
6. When using dissociated cells, the cells are plated onto the “bottom” collagen in a 50- μ L volume of 1X OptiMEM and allowed to adhere for 1 hr. The excess media is then gently removed with a 200- μ L micropipettor and the “top” collagen is applied.
7. The collagen in which the explants are washed will eventually accumulate DMEM and will start to gel if not replaced every three dishes or 18 explants.
8. As little as 10 μ L and as much as 200 μ L can be loaded onto the pump head with the micropipettor tip acting as a reservoir of solution.
9. There is no liquid media on top of the gels, because that would disrupt the stability of the gradient. Thus the incubator must have high humidity, which can be achieved by creating a gas-permeable humid chamber for the culture dishes within the incubator.

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Chapter 17

Assays for Chemotaxis and Chemoattractant-Stimulated TorC2 Activation and PKB Substrate Phosphorylation in *Dictyostelium*

Yoichiro Kamimura, Ming Tang, and Peter Devreotes

Summary

Chemotaxis is a highly coordinated biological system where chemoattractants trigger multiple signal transduction pathways which act in concert to bring about directed migration. A signaling pathway acting through PIP_3 , which accumulates at the leading edge of the cell, has been extensively characterized. However, chemotaxis still remains in cells depleted of PIP_3 , suggesting there are PIP_3 -independent pathways. We have identified a pathway involving TorC2-PKB R1 as well as another containing PLA2 activity that act in parallel with PIP_3 . Activation of PKB R1 , a myristoylated Protein Kinase B homolog, is dependent on TorC2 (Rapamycin-insensitive Tor complex 2) kinase but is completely independent of PIP_3 . In response to chemoattractant, PKBs rapidly phosphorylate at least eight proteins, including Talin B, PI4P 5-kinase, two RasGefs, and a RhoGap. These studies help to link the signaling pathways to specific effectors and provide a more complete understanding of chemotaxis.

Key words: Chemotaxis, *Dictyostelium discoideum*, PIP_3 , PKB, TorC2

1. Introduction

Investigations of *Dictyostelium discoideum* have been extremely useful in elucidating mechanisms of chemotaxis, motility, and cytokinesis. *D. discoideum* cells grow in their natural soil environment by consuming bacteria and yeasts. Known as a “social amoeba,” these protozoa change from a unicellular to a multicellular state upon nutrient starvation. In this process, cAMP is spontaneously released from central cells every 6 min and functions as a chemoattractant for the cells to aggregate. Within 24 h the multicellular structure undergoes morphogenesis and

differentiation, forming a fruiting body which contains spores that resist harsh conditions. The chemotaxis toward cAMP is easily and consistently reproducible under standard laboratory conditions. The following additional features of this organism increase its value as a model system for studies of eukaryotic chemotaxis. With a sequenced 34-Mbp genome size, the organism is genetically and biochemically tractable. For example, gene disruption by homologous recombination, restriction enzyme mediated insertional mutagenesis, and chemical mutagenesis are easily achieved. It is also simple and convenient to generate up to 3×10^{11} cells in suspension culture for biochemical assay and protein purification.

Accumulated evidence now indicates that a network of signaling pathways contribute to efficient chemotaxis (1–3) (Fig. 1). Chemoattractant binds to G-protein-coupled receptors uniformly distributed around the cell's periphery and cause activation of the heterotrimeric G-protein consisting of $G\alpha_2$ and $G\beta\gamma$. This activation leads to a rapid, and in most cases, transient burst of responses such as production of the second messengers PIP_3 , cGMP, and cAMP. PIP_3 accumulates locally at the front of cells by the

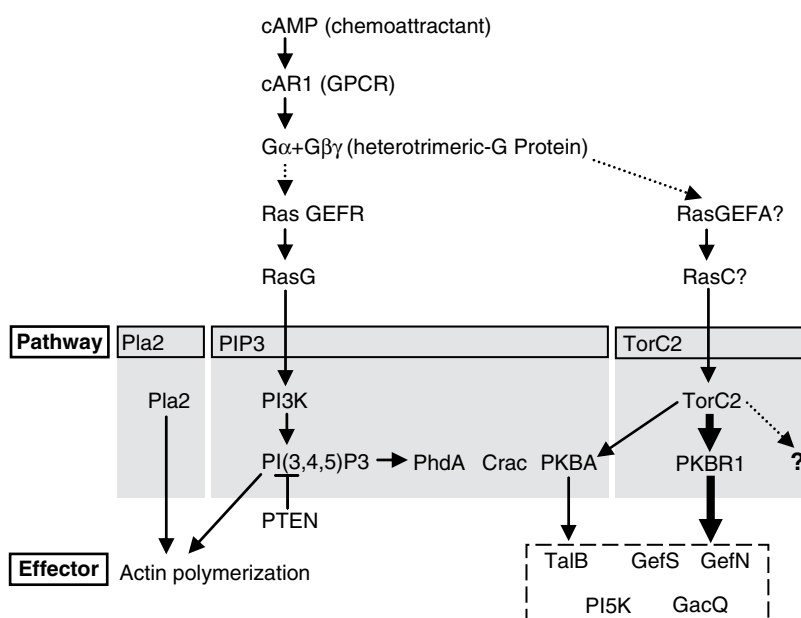


Fig. 1. Model of chemoattractant signaling pathways in *Dictyostelium discoideum*. The chemoattractant, cAMP, binds to the cAR1 and activates the heterotrimeric G-protein. The activation conveys signals via multiple pathways to successfully achieve chemotaxis. The indicated Ras proteins thought to be locally activated at the front of cells are suggested to regulate PI3K and TorC2 activities. PIP_3 production resulting from the countered reactions of PI3K and PTEN causes the recruitment of PH domain containing proteins, PhdA, Crac, and PKBA. Pla2 activity is required for the actin polymerization in parallel with PIP_3 pathway. TorC2 organizes the cAMP information through PKB and other undefined activities. PKB activity is composed of PKBR1, a more predominant activity and completely dependent on TorC2 for activation, and PKBA, a minor activity dependent on recruitment to PIP_3 and TorC2 (heavier or lighter arrows). Together these phosphorylate several substrates, for example, Talin B (TalB), RasGEFS (GefS), RasGEFN (GefN), PI5K, and Rho GAPQ (GacQ).

opposing actions of PI 3-kinase and PTEN which reversibly convert PI (4, 5) P2 to PI (3,4,5) P3. The information conveyed by the elevated PIP_3 is transmitted by recruitment of proteins containing PIP_3 -specific PH (Pleckstrin homology) domains such as CRAC (4), PhdA (5), and PKBA (6). Although misregulation of PIP_3 , as occurs in cells lacking PTEN greatly impairs chemotaxis (7), PI 3-kinase activity is not essential for chemotaxis (8). The search for PIP_3 -independent pathways has led to the isolation of PLA2 activity as an enhancer of PIP_3 -dependent chemotaxis (9). PLA2 activity appears to contribute to cell motility by regulating actin polymerization in parallel with PIP_3 .

Recently, we have shown that the TorC2-PKBR1 pathway plays an important role in chemotaxis and is independent of PIP_3 (10). *D. discoideum* has two PKB (Protein Kinase B) homologs, PKBA and PKBR1 (6, 11). Both proteins are structurally very similar to the mammalian PKBs, except for the membrane-binding domain at N-terminus. Like the mammalian enzymes, PKBA has a PIP_3 -specific PH domain which functions to recruit it to membrane-associated PIP_3 , while the myristoylated PKBR1 is evenly distributed on the membrane. These proteins require specific phosphorylations in the activation loop and in the hydrophobic motif for the full activity. A commercial antibody specific for the phosphorylated state of PKB substrates *in vivo* has provided evidence that PKBR1 is the major PKB activity and that PKBA makes a minor contribution. PiaA (pianissimoA), originally isolated in a forward genetic screen for chemotaxis and early development defects, is now recognized to be a subunit of TorC2 kinase (12, 13). Work in *Drosophila* and mammalian cells identified TorC2 as the kinase responsible for the phosphorylation of the hydrophobic motif of PKB (14). Consistent with this, the activity of PKBR1 depends on TorC2 phosphorylation of its hydrophobic motif. The TorC2-PKBR1 pathway is insensitive to PIP_3 depletion and is selectively activated at the cell's leading edge. The PIP_3 -independent PKBR1 and PIP_3 -dependent PKBA pathways converge with their overlapping substrates, including TalinB, RasGEFs, RhoGAP, PI5K, and others (10). Work is currently focused on determining which substrates are involved in signaling and cytoskeletal regulation for proper chemotaxis.

2. Materials

2.1. Cell Culture Media, Buffer, and Solutions

1. *HL5 medium*. 20 g maltose (or 10 g dextrose), 10 g proteose peptone, 5 g yeast extract, 0.51 g Na_2HPO_4 , 0.485 g KH_2PO_4 , H_2O to 1 L. Autoclave for sterilization (see Note 1).

2. *DB (Development buffer)*. 100 mL 10× phosphate buffer, 1 mL 2 M MgSO_4 , 0.2 mL 1 M CaCl_2 , H_2O to 1 L.
3. *10× Phosphate buffer*. 6.8 g KH_2PO_4 , 13.4 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, H_2O to 1 L (pH should be ~6.5 without adjustment).
4. *100 mM caffeine*. 1.94 g of caffeine, H_2O to 100 mL. Store at -20°C .
5. *PM (phosphate magnesium buffer)*. 100 mL 10× phosphate buffer, 1 mL 2 M MgSO_4 , H_2O to 1 L.
6. *Basal buffer*. 20 mM Tris-HCl of pH 8.0, 2 mM MgSO_4 .
7. *10 mM cAMP*. 0.0369 g cAMP sodium salt monohydrate, H_2O to 10 mL. Store at -20°C .

2.2. Micropipette Assay

1. Femtotip microinjection needles (Eppendorf).
2. Microinjector (Eppendorf).
3. Lab-Tek one-well glass chamber (Nalge Nunc, Naperville, PA).

2.3. Two-Drop Assay

1. 1% melted agar in H_2O (Difco Noble Agar, Becton Dickinson, Sparks, MD) (*see Note 2*).
2. *A drawn-out Pasteur pipette*. Soften a Pasteur pipette in a flame, withdraw it from the heat, and quickly pull both ends to a thin diameter, then break.

2.4. Western Blotting

1. *Precast gels*. Criterion Tris-HCl 4–15% gels (Bio-Rad, Hercules, CA).
2. PVDF membrane (Immobilon-P, Millipore, Bedford, MA) (*see Note 3*).
3. 3-MM Chr chromatography paper (Whatman, Maidstone, U.K.).
4. *Transfer Buffer*. 25 mM Tris base, 190 mM glycine, 20% (v/v) methanol.
5. *TBST (Tris-buffered saline with Tween)*. 20 mM Tris-HCl of pH 7.5, 137 mM NaCl, 0.1% Tween-20.
6. *Blocking buffer*. 5% (w/v) nonfat dry milk in TBST.
7. *Secondary antibody*. Antirabbit or antimouse IgG-conjugated HRP (horse radish peroxidase) (GE-Healthcare, Piscataway, NJ).
8. ECL (enhanced chemiluminescent) reagent (GE Healthcare).

2.5. Primary Antibodies for Western Blotting

All phosphospecific antibodies from Cell Signaling Technology, Danvers, MA.

1. Rabbit antiphospho PKB substrate monoclonal antibody (110B7). Use 1:2,500 dilution in TBST containing 5% (w/v) BSA; detect with antirabbit IgG-HRP.
2. Mouse antiphospho PDK docking motif monoclonal antibody (18A2). Use 1:2,000 dilution in TBST containing 5% (w/v) BSA; detect with antimouse IgG-HRP.

3. Rabbit antiphospho PKC (pan) monoclonal antibody (190D10). Use 1:2,000 dilution in TBST containing 5% (w/v) BSA; detect with antirabbit IgG-HRP.

2.6. Indirect Immunofluorescence

1. *Plasmid*. pJK1-R1-AKT-HA transfected cells are selected with G418. (see [Note 4](#)).
2. 18-mm coverslips and slideglasses (Fisher, Pittsburgh, PA).
3. *Fix solution* (see [Note 5](#)). Mix 0.2 g paraformaldehyde (see [Note 6](#)) and 5 mL of 20 mM PIPES Buffer (pH 6.0) in a 50-mL disposable tube, microwave in brief pulses until dissolved, and then cool quickly on ice to room temperature. Finally add 3.25 mL H₂O, 0.25 mL 2.5 M sucrose, and then 1.5 mL saturated picric acid solution (Fluka).
4. *Quenching solution*. 100 mM glycine in PBS.
5. *PBS (Phosphate-buffered saline)*. 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.4).
6. *Blocking solution*. 2% (w/v) BSA in TBS-TX100.
7. *TBS-TX100 (Tris-buffered saline with Triton X100)*. 20 mM Tris-HCl of pH 7.5, 137 mM NaCl, 0.1% Triton X100.
8. *Primary antibodies*. Rabbit anti phospho AKT(S473) polyclonal antibody (Cell Signaling Technology), diluted 1:50 in TBS-TX100. Anti HA polyclonal antibody (Zymed, South San Francisco, CA), diluted 1:100 in TBS-TX100.
9. *Secondary antibody*. Antirabbit IgG conjugated with rhodamine (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:100 in TBS-TX100.
10. *Mounting medium*. Vectashield (Vector Laboratories Inc., Burlingame, CA).

2.7. Immunopurification of PKB Substrates

1. *2× NP-40 lysis buffer*. 80 mM HEPES (pH 7.5), 100 mM NaF, 4 mM Na₃VO₄, 50 mM sodium pyrophosphate, 1% NP-40, 2× protease inhibitor complete EDTA free (Roche, Mannheim, Germany), 2% protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO).
2. Protein G-Sepharose (GE-Healthcare); wash twice with PBS containing 5 mg/mL BSA before use.
3. *Antibody*. Antiphospho PKB substrate antibody (Cell Signaling Technology).
4. *1× RIPA buffer*. Tris-HCl of pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× protease inhibitor complete EDTA free (Roche), 1% protease inhibitor cocktail (Sigma Aldrich).
5. 150 × 25 mm of tissue culture dish from (Becton Dickinson).

2.8. PIP₃ Detection Biochemically

1. Nucleopore Track-Etch membrane, 5 μ m pore size (Whatman).
2. *Syringes for cell lysis.* For each, fold a Nucleopore membrane in half and place it between the needle and barrel of a 1-mL disposable syringe.

2.9. Genes and Dictyostelium Data Base (DDB) Numbers

All genes referred to in these protocols are listed in **Table 1** together with their DDB numbers, which can be used to search dictybase (<http://www.dictybase.org>) to access a knowledge base for each gene, including sequence information, expression properties, relevant literature, and available resources.

3. Methods
3.1. Preparation of Chemotactically Competent Cells

D. discoideum shows reliable chemotaxis to cAMP at the early development stage, after 5-h starvation. These cells are typically used for chemotaxis analysis. This section describes the method for preparing the cells.

3.1.1. Starvation of Dictyostelium discoideum Cells

1. Culture axenically in HL5 medium at 22°C to a density of less than 5×10^6 cells/mL.
2. Centrifuge 1×10^8 cells for 5 min at $500 \times g$ and remove the supernatant. Wash with 50 mL of DB buffer twice.
3. Resuspend cells in 5 mL of DB at 2×10^7 cells/mL and rotate in a 50-mL flask for 1 h at 110 rpm.
4. Pulse with 60 nM of cAMP every 6 min for the next 4 h.

3.1.2. "Basalation" of Cells

1. Add caffeine to 5 mM.
2. Shake at 200 rpm for 30 min.
3. Centrifuge the cells for 5 min at $500 \times g$ at 4°C and remove the supernatant. Wash with 30 ml of ice-cold DB twice.
4. Resuspend at 2×10^7 cells/mL in DB and keep on ice before assay.

3.2. Under Buffer Assay

This section describes a method to assess cell aggregation in response to starvation with or without an inhibitor, such as LY294002 (see **Note 7**). This aggregation process includes chemotaxis and other cellular events. The method can also be applied to assess the defect in a mutant cell line.

1. Plate 1×10^5 cells in HL5 medium in wells of a 96-well plate.
2. Let them to adhere on the plate for 30–60 min.

Table 1
Genes and DDB (*Dictyostelium* Data Base) numbers

GENE	PKBA	PKBR1	PIAA	RIP3	TALB	GEFN
DDB #	DDB0191195	DDB0191365	DDB0185055	DDB0201626	DDB0191526	DDB0167277
GENE	GEFS	GACQ	PI5K	PDKA	PDKB	PI3K1
DDB #	DDB0191324	DDB0233774	DDB0234212	DDB0216243	DDB0216246	DDB0214949
GENE	PI3K2	PI3K4	PI3K5	PTEN	CRAC	PHDA
DDB #	DDB0191474	DDB0235157	DDB0235158	DDB0191093	DDB0191434	DDB0191446
GENE	PLA2A	CAR1	gα2 (GPA2)	gβ (GPBA)	gγ (GPGA)	RASC
DDB #	DDB0235269	DDB0185024	DDB0191237	DDB0252679	DDB0185201	DDB0214827
GENE	RASG	GEFA(ALEA)	GEFR			
DDB #	DDB0201663	DDB0191187	DDB0185198			

3. Aspirate the medium.
4. Add 200 μ L of DB with or without a drug.
5. Incubate at 22°C for about 18 h to form aggregates.
6. Take a picture using a dissection microscope (*see Fig. 2a*).

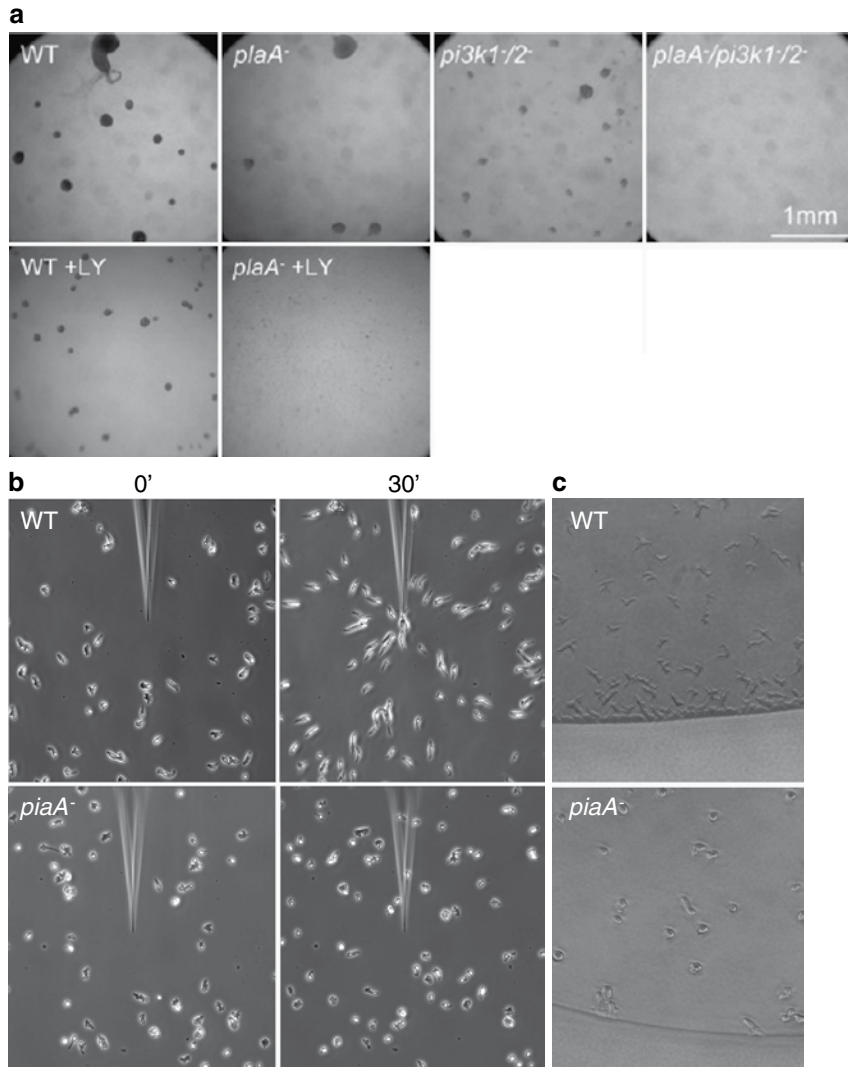


Fig. 2. Chemotaxis assays (a) Under buffer assay. In the upper panel, the indicated cells are starved in DB and allowed to make aggregates (*dark spots in a picture*). WT (wild-type), *Pla2A*⁻ (*plaA*⁻), and *PI3K1*^{-/2} (*pi3k1*^{-/2}) cells can aggregate, but the triple mutant cells (*plaA*⁻/*pi3k1*^{-/2}) cannot, suggesting that both *Pla2A* and *PI3K* function in parallel. The lower panel shows the effects of LY294002 (LY) as an inhibitor of *PI3K*. Consistent with the upper panel, LY prevents aggregation of only *pla2A*⁻ cells. (b) Micropipette assay. cAMP gradients are formed from the tip of the micropipette and the responses of WT and *piaA*⁻ cells are shown before (*left panel*) and 30 min after (*right panel*) the needle is placed. WT cells move toward the higher concentration of cAMP with a typically polarized morphology, but *piaA*⁻ cells abrogate chemotaxis as well as polarity. (c) Two-drop assay. The responses of WT (*upper panel*) and *piaA*⁻ cells (*lower panel*) are shown 30 min after the assay. A cAMP (not shown) and cell droplet are juxtaposed to each other. (Panels A and B reproduced from refs. 9 and 10, respectively, with permission from Elsevier Science.).

3.3. Chemotaxis Assays

This section describes two different methods to evaluate chemotaxis. These two methods are not necessarily identical in the degree of chemotaxis activity. It might be important to carry out several different assays to document subtle defects.

3.4. Micropipette Assay

1. Dilute competent cells to around $1\text{--}4 \times 10^5$ cells/mL in DB.
2. Disperse cells by pipetting several times or vortexing weakly.
3. Spot 20 μL on a chamber cover glass.
4. Wait for 10–15 min for cells to adhere; then gently add 3 mL of DB.
5. Load a Femtotip microinjection needle with 10 μM cAMP, connect the needle to a microinjector, lower the needle to touch the coverslip using a micromanipulator, and apply positive pressure (25 psi) with the microinjector.
6. Take photographs at 30-s intervals for 30 min (*see Note 8*) (*see Fig. 2b*).

3.4.1. Two-Drop Assay

1. Pour 10 ml of 1% melted agar in a 90-mm Petri dish 1 h before assay (*see Note 9*).
2. Dilute competent cells to about 1×10^6 cells/mL in DB.
3. Disperse cells by pipetting several times or vortexing weakly.
4. Spot a 3-mm-diameter drop of cell suspension ($\sim 200\text{--}500$ cells/drop) on the agar surface using the fine tip of a drawn-out Pasteur pipette and capillary action. Spots of DB with or without 0.01, 0.1, or 1 μM cAMP are placed 3 mm from the cell spots. Wait for 30–60 min.
5. Evaluate positive chemotaxis under a microscope by viewing each spot. Positive chemotaxis is scored when cells move to the edge of the drop toward the nearby cAMP and away from the far edge (*see Note 10*) (*see Fig. 2c*).

3.5. Detection of PIP_3 Production

This section describes the detection of PIP_3 products, the output of the PI3K pathway, by imaging and by biochemical analysis. A PIP_3 -specific PH domain from either CRAC or PKBA is typically used as a biosensor.

3.5.1. PIP_3 Detection by a Biosensor, PH-GFP (Fluorescent Microscope)

PIP_3 production following addition of uniform chemoattractant or spatial localization in cells under a variety of conditions is detected by one of the PIP_3 -specific PH domain fused with a fluorescent protein (6, 7).

1. Starve cells expressing this biosensor to chemotactic competence and uniformly stimulated with cAMP at a final concentration of 1 μM .
2. Capture images at 2-s intervals for 1–2 min.

3. To visualize the localized accumulation of PIP₃, cells are placed on the chamber coverslip and observed under fluorescence microscope doing chemotaxis toward spontaneously secreted cAMP or toward a gradient from a micropipette.

**3.5.2. PIP₃ Detection
by a Biosensor, PH-GFP
(Biochemically)**

1. Starve cells expressing PH-GFP at 2×10^7 cells/mL in 5 mL and basalate with caffeine as described in **Subheading 3.1.1**.
2. Wash cells with 30 mL of ice-cold PM twice.
3. Suspend cells in 1.25 mL of PM (i.e., 8×10^7 cells/mL).
4. Transfer the cells to a 5-ml disposable plastic beaker and add 100 μ M of cAMP to a final concentration of 1 μ M.
5. Take 100 μ L of cells at time points of 0, 5, 20, 60 s. (see **Note 11**)
6. Transfer into a 1-mL of disposable syringe containing 100 μ L of basal buffer and lyse through 5- μ m membrane into a microcentrifuge tube containing 1 mL PM on ice.
7. Microcentrifuge at full speed for 1 min at 4°C and aspirate the supernatant.
8. Suspend the pellet in 50 μ L of 1× SDS sample buffer and boil them for 5 min.
9. Run SDS PAGE followed by western blotting with α -GFP antibody as a primary antibody.

**3.6. Detection of PKB
and TorC2 Activity**

This section describes methods to biochemically and cytologically assess PKB and TorC2 activity using the following phospho-specific antibodies. First, a phospho PKB substrate antibody used to detect the phosphorylated state of pp350, pp200, pp180 (GefS), pp110 (GefN and PI5K), and pp65/67 (GacQ) and other substrates of PKB. Second, a phospho PDK docking motif antibody used to detect the phosphorylated state of the hydrophobic motif of PKBR1 (T470). For PKBR1 the extent of this phosphorylation correlates strongly with the activation state of TorC2. Third, a phospho PKC (pan) antibody used to detect the phosphorylation state of the activation loops of PKBR1 (T309) and PKBA (T278). Evidence from other model systems suggests that these phosphorylations would be catalyzed by a PDK homolog and essential for their activities. Two PDK homologs are present in *D. discoideum*. Fourth, antiphospho AKT (S473) can be used to detect activation of TorC2 in cells expressing a chimeric protein where the PH domain of human AKT is replaced with the myristoylated N-terminal of PKBR1.

**3.6.1. cAMP Stimulation
and Sample Preparation**

1. Transfer 0.5-mL competent cells on ice (see **Note 12**) at a density of 2×10^7 cells/mL to a 5-mL disposable plastic beaker shaking at 150 rpm (see **Note 13**). Within 2 min, add 100 μ M of cAMP to final concentration of 1 μ M.

2. Transfer 40 μ L of cells at time points of 10, 20, 30, 60, 120, 180 s into microcentrifuge tubes containing 10 μ L of 5 $\times$ SDS sample buffer.
3. Quickly move tube to a hot-block at 95°C for 5 min.

3.6.2. Western Blotting

1. Load wells of precast gels with 2.5 μ L of sample (4×10^4 cells) for antiphospho PKB substrate antibody and 5 μ L (8×10^4 cells) for antiphospho PDK docking motif antibody and antiphospho PKC (pan) antibody.
2. Run gels by electrophoresis at 150 V for 85 min.
3. To transfer proteins to a PVDF membrane, place a pad and two sheets of 3-MM paper wetted with the transfer buffer on the black (cathode -) side of the cassette holder. Place the gel on the 3-MM paper and lay the PVDF membrane on top of it. Remove bubbles between the gel and the PVDF and put two more sheets of 3-MM paper and a pad.
4. Place the sandwich into the transfer tank such that the PVDF membrane is between the gel and the anode (+) and fill the cold transfer buffer.
5. Turn on the system for 80 min at 75 mV in the cold room.
6. After the transfer, rinse the PVDF membrane with TBST twice quickly.
7. Incubate the membrane in 50 ml of blocking buffer for 1 h at room temperature.
8. Discard the blocking buffer and rinse the membrane with TBST twice quickly.
9. Incubate the membrane with a primary antibody at 4°C overnight.
10. Remove the primary antibody, rinse the membrane with TBST twice quickly, and further wash with TBST for 10 min, 5 min, and 5 min at room temperature.
11. Incubate the membrane with a secondary antibody for 1 h at room temperature.
12. Remove the secondary antibody, rinse the membrane with TBST twice quickly, followed by one 10 min and four 5 min washes with TBST at room temperature.
13. Prepare the ECL reagent and incubate the membrane with it for 1 min.
14. Remove the ECL reagent and wrap the membrane with plastic wrap.
15. Expose the membrane to X-ray film in the cassette for a suitable time.

16. To confirm the loading of proteins, stain the membrane in CBB solution for couple of minutes and destain it in 50% methanol for suitable time, typically a few minutes. Place the membrane on a bench to let it dry at room temperature.

As shown in **Fig. 3a**, the phospho PKB substrate antibody stains few bands in competent cells prior to addition of cAMP. Following cAMP stimulation, about ten prominent bands rapidly

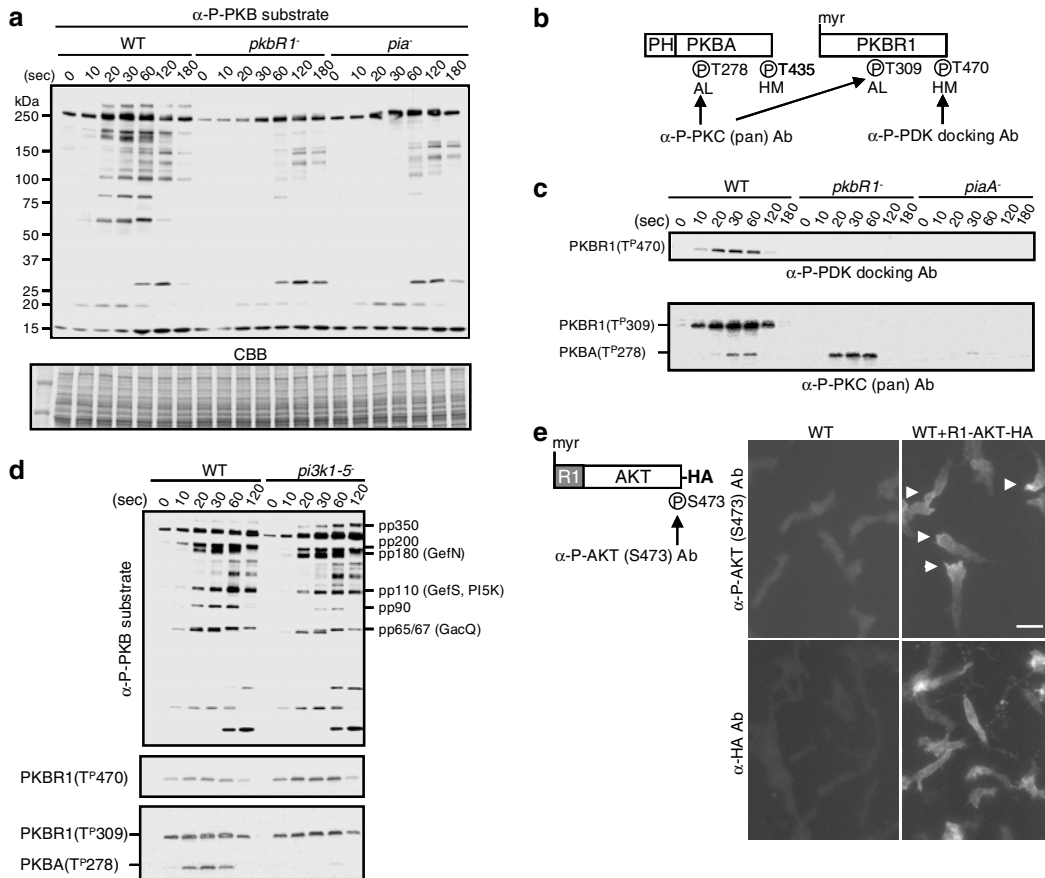


Fig. 3. Assays to detect TorC2 and PKB activities. **(a)** The in vivo PKB activity is evaluated by antiphospho PKB substrate antibody (α -P-PKB substrate). The bands of pp350, pp200, pp180, pp110, pp90, and pp65/67 in WT cells are dependent on the activities of PKBR1 and PiaA, a subunit of TorC2. CBB (coomassie brilliant blue) staining is for the loading control. **(b)** The schematic structures of PKBA and PKBR1 are shown. The phosphorylation sites (P) can be detected by the antibodies, antiphospho PDK docking motif antibody (α -P-PDK docking Ab), and antiphospho PKC (pan) antibody (α -P-PKC (pan) Ab). The labels AL and HM refer to activation loop and hydrophobic motif. **(c)** The *upper panel* shows the phosphorylation at T470 of PKBR1 in WT, $pkbR1^-$, $piaA^-$ cells by α -P-PDK docking Ab. The *lower panel* shows the phosphorylations at T278 of PKBA and T309 of PKBR1 in WT, $pkbR1^-$, $piaA^-$ cells by α -P-PKC (pan) Ab. **(d)** The PKB activity, the phosphorylation of T470 of PKBR1 by α -P-PDK docking Ab, and the phosphorylations of T278 of PKBA and T309 of PKBR1 by α -P-PKC (pan) Ab are compared in between WT and $pi3k1-5^-$ cells. **(e)** The schematic structure of R1-AKT-HA is shown with the phosphorylation sites (P) that are detected by the antiphospho AKT (S473) antibody (α -P-AKT (S473) Ab). In the *right panel*, chemotaxing cells are stained with α -P-AKT (S473) Ab to detect the phosphorylation of S473 or α -HA Ab for the localization of R1-AKT-HA. Arrow heads show localized staining. The scale bar represents 10 μ m. (Reproduced from ref. 10 with permission from Elsevier Science.).

appear and display characteristic time courses, eventually subsiding over the next several minutes. Seven or eight of these bands are specific PKB substrates which are greatly decreased in cells lacking PKB activity such as *pkbRI*⁻, *pkbRI*⁻/*pkbA*⁻, and *pia*⁻. These bands reappear when the appropriate proteins are expressed in the null mutants. A few of the bands (pp250, pp30, and pp23) are not dependent on PKB activity and are presumably substrates for other kinases with the consensus motif RXXXS/T.

As shown in **Fig. 3b, c**, the phospho PDK docking motif antibody, very specifically detects the phosphorylation state of the hydrophobic motif of the PKBR1. This phosphorylation is completely absent in competent cells prior to stimulation. Following addition of cAMP, it increases rapidly, peaks at 30 s, and disappears within 2 min. This phosphorylation is completely absent in cells lacking PiaA and substantially decreased in cells lacking Rip3 (data not shown), indicating that it is a TorC2 substrate.

As shown in **Fig. 3c**, the phospho PKC (pan) antibody specifically detects the activation loop phosphorylations of PKBA and PKBR1. The time courses of these phosphorylations closely follow those of the hydrophobic motif (see **Fig. 3c**). The phosphorylation of PKBA (T278) is abolished in cells lacking PIP₃ production such as *pi3kI*⁻/*5*⁻ cells (see **Fig. 3d**) and substantially reduced in *piaA*⁻ cells (see **Fig. 3c**). Interestingly, however, the phosphorylation of PKBR1 (T309) is unaffected by the absence of PIP₃ (see **Fig. 3d**). It does absolutely require a prerequisite phosphorylation at T470 since T309 is not phosphorylated in T470A versions of PKBR1 or in *piaA*⁻ cells (see **Fig. 3c**). These observations show that the activation of PKBR1 (T309) depends on TorC2 and not PIP₃, while activation of PKBA (T278) depends on both TorC2 and PIP₃. This blotting with the activation loop antibody can be used on any cell line to rapidly assess activation of TorC2 as well as PI3K. The antibody is also effective in detecting TorC2 activity cytologically in cells overexpressing PKBR1.

3.6.3. Indirect Immunofluorescence

1. Place 18-mm coverslips on parafilm.
2. Put 5×10^5 competent cells expressing R1-AKT-HA in 500 μ L DB on the coverslip and allow cells to initiate chemotaxis for 20–30 min at room temperature.
3. Place 500 μ L fix solution on the parafilm and transfer the coverslip into this solution followed by 30-min incubation at room temperature.
4. Transfer the coverslip into 500 μ L of quenching solution on the parafilm followed by 10-min incubation at room temperature.
5. Wash the coverslip with PBS twice.

6. Permeabilize and block cells by incubation in the blocking solution for 30 min at room temperature.
7. Remove the blocking solution and place the coverslip cells facing down on 100 μ L of the primary antibody solution, either anti Phospho AKT(S473) or anti HA, on the parafilm followed by overnight incubation in the moist chamber at 4°C.
8. Wash the coverslip in 3 mL of TBS using 6-well plate for 5 min three times.
9. Incubate with 100 μ L of the secondary antibody (as was done for the primary antibody step) for 1 h at room temperature in the dark.
10. Wash the coverslip in 3 mL of TBS using 6-well plate for 5 min three times.
11. Dip the coverslip once in deionized water.
12. Invert the coverslip cells attached side down and put into a drop of mounting medium on a slide glass (*see* [Note 14](#)).
13. Observe the sample under the fluorescence microscope by excitation at 543 nm. (*see* [Fig. 3e](#))

The TorC2-PKBRI pathway is selectively activated at the leading edge of chemotaxing cells. The R1-AKT-HA chimeric protein, where the PH domain of human AKT is replaced with N-terminus PKBRI containing the myristoylation site, is phosphorylated at S473 in the hydrophobic motif in response to cAMP stimulation. This response is completely dependent on TorC2 activity and does not occur in cells lacking *PiaA*. As shown in [Fig. 3e](#), indirect immunofluorescence of a cell expressing the R1-AKT-HA by antiphospho AKT(S473) antibody can be used to visualize the activation of TorC2 cytologically. Prior to stimulation there is little or no staining of cells. In highly polarized cells the staining is found selectively at the leading edge.

3.7. Purification of Substrates of PKB

This section describes the purification of PKB substrates.

1. After baselining (*see* [Subheading 3.1.1](#)), cells are washed with 15 mL of ice-cold PM buffer twice.
2. Stimulate cells with or without 1 μ M cAMP for 30 s.
3. After stimulation, lyse cells with an equal volume of 2 $\times$ NP-40 lysis buffer on ice for 5 min.
4. Centrifuge the cell extracts for 5 min at full speed in microcentrifuge at 4°C.
5. Transfer the supernatant to a new microcentrifuge tube.
6. Incubate the supernatant with prewashed protein G-Sepharose (*see* [Subheading 2.7, item 2](#)) for 1 h at 4°C.

7. Centrifuge at $500 \times g$ for 1 min and transfer the supernatant into a new microcentrifuge tube.
8. Add antiphospho PKB substrate antibody (110B7) (1% volume of total cell extracts) and incubate it overnight at 4°C .
9. Add prewashed protein G-Sepharose and incubate for further 1 h at 4°C .
10. Centrifuge at $500 \times g$ for 1 min and wash the beads with $1\times$ NP-40 buffer four times (twice quickly and twice for 15 min).
11. Repeat **step 10** using $1\times$ RIPA buffer.
12. Elute the proteins by boiling beads in $1\times$ SDS sample buffer.
13. Separate proteins by SDS-PAGE on 18-well precast gels.
14. Visualize proteins by silver staining (Silver Quest Silver-staining Kit; Invitrogen) in a 150×25 mm of tissue culture dish.

4. Notes

1. Antibiotics, such as streptomycin, may be added to prevent contamination.
2. Noble agar can be substituted for hydrophobic agar used in the original protocol ([15](#)).
3. The PVDF membrane is quickly prewetted with 100% methanol followed by 2-min soak in H_2O and equilibrated for at least 5 min in the transfer buffer.
4. For AX3 strains, $20 \mu\text{g}/\text{mL}$ of G418 is used.
5. Fixative solution should be prepared freshly.
6. Measure in the hood. Do not inhale.
7. LY294002 is dissolved in DMSO (Dimethyl sulfoxide). Since DMSO affects cell differentiation, the comparable concentration of DMSO is used as a control.
8. For a large field, a $10\times$ phase objective lens is used.
9. Do not dry the plate too much; otherwise, a droplet cannot be maintained during assay.
10. Score at least eight spots per test concentration.
11. Take duplicate 0-s samples for basal activity.
12. If cells are not maintained on ice, they spontaneously secrete and respond to cAMP within 7 min.

13. A styrofoam rack for 50-mL tubes makes a convenient holder of multiple beakers.
14. The sample is stable at 4°C for at least 1 month.

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Chapter 18

Biochemical Responses to Chemoattractants in *Dictyostelium*: Ligand–Receptor Interactions and Downstream Kinase Activation

Xin-Hua Liao and Alan R. Kimmel

Summary

Dictyostelium discoideum is one of the most facile eukaryotic systems for the study of chemotactic response to secreted chemical ligands. *Dictyostelium* grow as individual cells, using bacteria and fungi as primary nutrient sources; during growth, *Dictyostelium* moves directionally toward folate, a bacterial byproduct. Upon nutrient depletion *Dictyostelium* initiates a multicellular development program characterized by the production and secretion of cAMP. Cell surface receptors specifically recognize extracellular cAMP, which serves as both a morphogen to promote development and a chemoattractant to organize multicellularity. We discuss several approaches for the study of ligand–receptor interaction, with focus on affinity class determination and quantification of ligand binding sites (i.e., receptors) per cell. We further present examples for the application of biochemical assays to characterize the ligand-induced kinase activation of PI3K, GSK3, and ERK2.

Key words: Receptor affinities, cAMP, PIP₃, ERK, GSK3, Folate, G proteins

1. Introduction

Dictyostelium grow as single cells, but enter a multicellular developmental cycle upon nutrient depletion (1); *Dictyostelium* exhibit strong chemotactic response at all stages of its life cycle (1–3). During single-cell growth, *Dictyostelium* utilizes folic acid as a chemoattractant toward nutrient sources. Upon starvation, multicellular development is initiated and cells mobilize a new class of cell surface chemoattractant receptors. These are the receptors for secreted extracellular cAMP which, for *Dictyostelium*, is both a chemoattractant and a morphogen. Four different cell

surface receptors for cAMP, CAR1, CAR2, CAR3, and CAR4 are expressed during *Dictyostelium* development (4–9). Their stimulations at distinct stages are required to direct cell movement for the formation of the multicellular organisms and to activate intracellular signaling pathways that are essential for development.

We first discuss methods to characterize cAMP binding to cell surface receptors (10–12). We detail two types of binding assays. One permits the determination of cAMP binding under physiological conditions, including the estimation of relative K_d as well as the quantification of receptor numbers per cell. Binding under nonphysiological conditions permits analyses of the very low affinity receptors, which are otherwise unobserved under standard binding conditions. Both approaches can be adapted to determine the relative binding affinity of the receptors for various analogs of cAMP (13). This is useful for identifying analogs with high (or low) affinity for the cell surface CARs, but opposite affinity for the regulatory subunit of protein kinase A, an intracellular cAMP binding protein.

As cAMP receptors are members of the GTP-binding protein coupled class of receptors, they exhibit highest affinity for cAMP when complexed with $G\alpha\beta\gamma$. Upon cAMP binding, the heterotrimeric G proteins become uncoupled and the receptors exhibit an affinity decrease. Methods are presented that measure the impact of GTP-binding on cAMP–receptor interaction (14–16).

Three different kinase assays are also presented. Phosphoinositide 3-kinase, PI3K, converts phosphatidylinositol (4,5)-bisphosphate [$PI(4,5)P_2$] to phosphatidylinositol (3,4,5)-trisphosphate [$PI(3,4,5)P_3$]. cAMP treatment rapidly stimulates $PI(3,4,5)P_3$ production in *Dictyostelium* in cell lysates (16, 17), but as receptor response becomes desensitized to continuous stimulation of saturating levels of cAMP, $PI(3,4,5)P_3$ levels decline to prestimulus levels. This is due to a decline in PI3K activity as well as the phosphatase action of PTEN, which converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$. Protein kinase GSK3 activity is measured directly in lysates following a cAMP stimulus using a specific protein substrate (18, 19); controls treated with the GSK3-inhibitor LiCl are used to further establish specificity. Finally, we discuss the activation of the MAP kinase ERK2 by both cAMP and folate (20–23).

2. Materials

2.1. Solutions

1. Growth Medium.
2. *PM buffer*. 5 mM Na_2HPO_4 , 5 mM KH_2PO_4 , 2 mM $MgSO_4$, pH 6.2.

3. *cAMP*. 1 mM cAMP in PM, dilute as needed.
4. [^3H]-*cAMP*. 1 mCi/ml [2,8- ^3H] cAMP in EtOH:H₂O (1:1) (33.5 Ci/mmol; 30 μM ; 2.2×10^6 dpm/ μl).
5. 1% SDS.
6. "Saturated" Ammonium Sulfate Prepare 4 M (NH₄)₂SO₄ solution, filter, and dilute to 98%.
7. 300 μM GTP γS .
8. 0.1 M Caffeine; for most consistent results, prepare this fresh.
9. *GSK3 lysis buffer*. 0.5% NP40, 10 mM NaCl, 20 mM PIPES of pH 7.0, 5 mM EDTA, 50 mM NaF, 0.1 mM Na₃VO₄, 0.05% 2-mercaptoethanol, 5 mg/ml benzamidine, 5 mg/ml aprotinin.
10. *GSK3 reaction assay buffer*. 50 mM HEPES of pH 7.5, 4 mM MgCl₂, 0.5 mM EGTA, 2 mM DTT, 100 mM [γ - ^{32}P]-ATP at 1 Ci/mmol.
11. 15 mM phosphoric acid.
12. 500 mM LiCl.
13. [γ - ^{32}P]-ATP (3,000 Ci/mmol)
14. 1N HCl.
15. Thin-layer chromatography (TLC) solutions:
 - (a) 1.2% potassium oxalate in H₂O:methanol (3:2).
 - (b) Chloroform:methanol (1:1).
 - (c) Methanol:1N HCL (1:1).
 - (d) Chloroform:methanol (2:1).
 - (e) H₂O:methanol (3:2).
 - (f) Chloroform:acetone:methanol:acetic acid:H₂O (30:12:10:9:6).
 - (g) Iodine crystals
16. 5 mM folic acid; prepared fresh every 2 weeks. Neutralize as needed with 1N NaOH.

2.2. Supplies

1. Nuclepore membrane, 5 μm (Whatman).
2. P81 phosphocellulose paper (Whatman).
3. GSK3 peptide substrate RRRPASVPPSPSLSRHSpSHQRR, where pS is phosphoserine.
4. Silica gel 60 TLC plate (VWR).
5. *Protein gel-loading buffer*. NuPAGE[®] LDS Sample Buffer (4 $\times$) (Invitrogen).
6. Precast protein gel electrophoresis: NuPAGE Novex 4–12% Bis-Tris Gel 1.0 mm, 15 well (Invitrogen).

7. Gel running buffer: NuPAGE MES SDS Running Buffer (Invitrogen).
8. Actin antibody (Santa Cruz Biotechnology #SC-1616).
9. pThr202/pThr204-ERK antibody (Cell signaling Technology #9101).
10. Nitrocellulose membrane, 0.45 μ m pore size (Invitrogen).

3. Methods

3.1. Development of Aggregation-Competent Dictyostelium in Shaking Culture (See Note 1)

1. Grow cells to $\sim 2\text{--}5 \times 10^6$ cells/ml at 20°C.
2. Centrifuge cells at 20°C for 5 min at $1,500 \times g$.
3. Wash cells once with PM at 20°C.
4. Resuspend cell pellet in PM at 2×10^7 cells/ml.
5. The cell suspension is then shaken at 100–125 rpm at 20°C.
6. To develop cells by cAMP pulsing, add cAMP to an immediate (within 10 s) final concentration of 75 nM every 6 min for 6 h (24). This can be automated by connecting a peristaltic pump to a programmable on/off timer; for a 6-ml cell suspension culture, one can add 100 μ l of a 4.5 μ M cAMP stock solution. After 6 h, the cell volume will have doubled.

3.2. Binding of Surface Cell Receptors for cAMP in Phosphate Buffer (See Note 2)

1. Develop cells in shaking culture, as per Subheading 3.1.
2. Centrifuge cells at 20°C for 5 min at $1,500 \times g$.
3. Wash cells once with 10 mM phosphate buffer, pH 6.5 at 20°C.
4. Resuspend cell pellet in 10 mM phosphate buffer, pH 6.5 with 10 mM DTT at 2×10^8 cells/ml.
5. Add 100 μ l of 0.1 μ M [^{3} H]-cAMP (700,000 dpm) in 10 mM phosphate buffer to a 100 μ l cell aliquot. To determine nonspecific [^{3} H]-cAMP binding, include controls that contain 10 μ l of 1 mM unlabeled cAMP.
6. Mix and incubate at 4°C for 5 min.
7. Centrifuge at 4°C for 2 min at $14,000 \times g$ and remove supernatants.
8. Dissolve the pellets in 100 μ l 1% SDS and determine the bound [^{3} H]-cAMP by scintillation counting.
9. Calculate numbers of cAMP receptors per cell from the obtained dpm/cell number value using the specific activity of [^{3} H]-cAMP (33.5 Ci/mmol) and corrected for nonspecific background binding of [^{3} H]-cAMP.

3.3. Binding Affinity of Surface Cell Receptors for cAMP in Phosphate Buffer (See Note 2)

1. To determine receptor affinity for cAMP in phosphate buffer, follow steps 1–4 of Subheading 3.2.
2. Add 100 μ l containing varying concentrations of [^{3} H]-cAMP from 1 pM to 1.0 μ M in 10 mM phosphate buffer to a 100- μ l cell aliquot, varying concentrations by threefold differences. To determine nonspecific [^{3} H]-cAMP binding, controls are included that contain 10 μ l of 1 mM unlabeled cAMP.
3. Follow steps 6–8 of Subheading 3.2.
4. Several methods can be used to obtain relative affinity values. In the Scatchard plot the specific binding (X -axis) is plotted vs. the specific binding divided by free radioligand concentration (Y -axis). The X intercept will give the approximate number of receptors per cell. The negative reciprocal of the slope will give the relative affinity for cAMP as K_d .

3.4. Relative Binding Affinities for cAMP Analogs in Phosphate Buffer (See Note 2)

1. To determine the relative affinity of the cell surface cAMP receptors for a cAMP analog, follow steps 1–4 of Subheading 3.2.
2. Add 100 μ l of 0.1 μ M [^{3} H]-cAMP (700,000 dpm) in 10 mM phosphate buffer to a 100- μ l cell aliquot, but include varying concentrations of the analog; concentrations should range from 1 pM to 1 mM, varying by threefold differences. To determine nonspecific [^{3} H]-cAMP binding, controls are included that contain 10 μ l of 1 mM unlabeled cAMP.
3. Follow steps 6–8 of Subheading 3.2.
4. A semilog plot of analog concentration (X -axis) vs. bound dpm (Y -axis) can be used to determine relative affinity for a cAMP analog.

3.5. Binding of Surface Cell Receptors for cAMP in the Presence of Saturated $(\text{NH}_4)_2\text{SO}_4$ (See Note 2)

1. Develop cells in shaking culture, as per Subheading 3.1.
2. Centrifuge cells at 20°C for 5 min at $1,500 \times g$.
3. Wash cells once with 10 mM phosphate buffer at 20°C.
4. Resuspend cell pellet in 10 mM phosphate buffer with 10 mM DTT at 2×10^8 cells/ml.
5. Add 100 μ l of 0.1 μ M [^{3} H]-cAMP (700,000 dpm) in 10 mM phosphate buffer to a 100- μ l cell aliquot. To determine nonspecific [^{3} H]-cAMP binding, controls are included that contain 10 μ l of 1 mM unlabeled cAMP.
6. Mix and Incubate at 4°C for 30 s.
7. Add 1.2 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ and incubate for 5 min at 4°C.
8. Centrifuge at 4°C for 2 min at $14,000 \times g$ and remove supernatants.

9. Dissolve the pellets in 100 μ l 1% SDS and determine the bound [^{3} H]-cAMP by scintillation counting.
10. Calculate numbers of cAMP receptors per cell from the obtained dpm/cell number value using the specific activity of [^{3} H]-cAMP (33.5 Ci/mmol) and corrected for nonspecific background binding of [^{3} H]-cAMP.

3.6. Binding Affinity of Surface Cell Receptors for cAMP in the Presence of Saturated $(\text{NH}_4)_2\text{SO}_4$ (See Note 2)

1. To determine the relative affinity of the cell surface cAMP receptors for cAMP follow **steps 1–4 of Subheading 3.5**.
2. Add 100 μ l containing varying concentrations of [^{3} H]-cAMP from 1 pM to 1 mM in 10 mM phosphate buffer to a 100- μ l cell aliquot, varying then concentrations by threefold differences. To determine nonspecific [^{3} H]cAMP binding, controls are included that contain 10 μ l of 1 mM unlabeled cAMP.
3. Follow **steps 6–9 of Subheading 3.5**.
4. Several methods can be used to obtain relative affinity values. In the Scatchard plot the specific binding (X -axis) is plotted vs. the specific binding divided by free radioligand concentration (Y -axis). The X intercept will give the approximate number of receptors per cell. The negative reciprocal of the slope will give the relative affinity for cAMP as K_d .

3.7. Relative Binding Affinities for cAMP Analogs in the Presence of Saturated $(\text{NH}_4)_2\text{SO}_4$ (See Note 2)

1. To determine the relative affinity of the cell surface cAMP receptors for a cAMP analog, follow **steps 1–4 of Subheading 3.5**.
2. Add 100 μ l of 0.1 μ M [^{3} H]-cAMP (700,000 dpm) in 10 mM phosphate buffer to a 100- μ l cell aliquot, but include varying concentrations of the analog; concentrations should range from 1 pM to 1 mM, varying by threefold differences. To determine nonspecific [^{3} H]-cAMP binding, controls are included that contain 10 μ l of 1 mM unlabeled cAMP.
3. Follow **steps 6–9 of Subheading 3.5**.
4. A semilog plot of analog concentration (X -axis) vs. bound dpm (Y -axis) can be used to determine relative affinity for a cAMP analog.

3.8. GTP γ S Inhibition of cAMP Receptor Binding

1. Develop cells in shaking culture, as per **Subheading 3.1**.
2. After 6 h, centrifuge cells at 20°C for 5 min at $1,500 \times g$.
3. Resuspend cell pellet in PM at 1×10^7 cells/ml.
4. Add caffeine to 2 mM and shake cells at 200 rpm for 30 min at 20°C.
5. Wash cells twice with ice-cold PM and resuspend at 1×10^7 cells/ml in PM; leave cells on ice.

6. Lyse cells by passage through a 3–5 μm Nuclepore filter.
7. Pellet membranes by centrifugation for 2 min at $14,000 \times g$ at 4°C .
8. Wash membranes once in PM at 4°C .
9. Resuspend membranes in the original PM volume used in **step 5**; leave on ice.
10. Mix 70 μl of membranes with 10 μl of 50 nM [^3H]-cAMP-50 mM DTT in the presence or absence of 10 μl of 300 μM GTP γ S at 4°C . To determine nonspecific [^3H]-cAMP binding, incubate control membranes as earlier with the addition of 10 μl of 1 mM unlabeled cAMP. Adjust final volumes for each reaction to 100 μl with H_2O ; final concentrations will be 5 mM for DTT, 5 nM for [^3H]-cAMP, 30 μM for GTP γ S, and 100 μM for unlabeled cAMP.
11. Incubate for 5 min on ice.
12. Centrifuge membranes for 2 min at $14,000 \times g$, and remove the supernatants.
13. Dissolve the pellets in 100 μl 1% SDS and determine the bound [^3H]-cAMP by scintillation counting.
14. Determine GTP γ S inhibition of cAMP binding by comparing values obtained in the presence or absence of GTP γ S and corrected for nonspecific background binding of [^3H]-cAMP.

3.9. cAMP Stimulation of Developed Cells

1. Develop cells in shaking culture, as per **Subheading 3.1**.
2. After 6 h, centrifuge cells at 20°C for 5 min at $1,500 \times g$.
3. Resuspend cell pellet in PM at 1×10^7 cells/ml.
4. Add caffeine to 2 mM and shake cells at 200 rpm for 30 min at 20°C .
5. Wash cells twice with ice-cold PM and resuspend at 8×10^7 cells/ml in PM; leave cells on ice.
6. Aliquot 2 ml of cells into a small plastic cup and shake at 200 rpm at 20°C .
7. Immediately stimulate cells with cAMP to a final concentration of 10 μM ; for each 1 ml of cell suspension, add 10 μl of a 1-mM cAMP stock. By varying the cAMP concentration it is possible to determine a dose response curve.

3.10. cAMP-Stimulated Synthesis of PI(3,4,5) P_3 [Phosphatidylinositol (3,4,5)-Trisphosphate]

1. Lyse 1 ml of cAMP-stimulated cells, as per **Subheading 3.9**, by passage through a 3–5 μm Nuclepore filter into a tube containing 20 μCi [$\gamma\text{-}^{32}\text{P}$]ATP.
2. Incubate lysates for various times at 20°C and take 100 μl aliquots at 5–15 s intervals.

3. Add 1 ml of 1N HCl to each aliquot.
4. Extract lipids with 2 ml chloroform:methanol (1:1).
5. Centrifuge the samples at $1,000 \times g$ for 5 min.
6. Collect the lower phase and further extract it with 2 ml methanol:1N HCl (1:1).
7. Centrifuge the samples at $1,000 \times g$ for 5 min and then collect and dry the lower phase under nitrogen gas.
8. Resuspend the dried lipid samples in 30 μ l of chloroform:methanol (2:1).
9. To separate lipids by thin-layer chromatography, first prepare a silica gel 60 TLC plate by prerunning overnight in 1.2% potassium oxalate in H_2O :methanol (3:2) and then drying at $100^\circ C$ for 3 min.
10. Spot 10 μ l of each lipid sample on the prepared TLC plate. 1 μ g of PIP, PIP₂, and PIP₃ (Sigma) can be used as mobility standards.
11. Run the plate in chloroform:acetone:methanol:acetic acid: H_2O (30:12:10:9:6).
12. When the solvent front reaches the top of the plate, dry the plate and expose it to either an X-ray film or a phosphorimager detection screen. Relative band intensities are then quantified. To visualize the standards, place the TLC plate in a tank that had been equilibrated 1–2 days with a few crystals of iodine. Lipids will appear as brownish spots within 1 h, but the color will fade once the plate is removed from the iodine vapor.
13. Bands can also be excised and radioactivity determined directly by scintillation counting.
14. Maximum levels of PI(3,4,5)P₃ are observed at 5 s, with decreasing levels observed as the PI3K pathway adapts.

3.11. cAMP Stimulation of GSK3 Activity

1. Take 50 μ l of prestimulus (0 time) control cells, as well as, cells at 15–30 s intervals following stimulation with 10 μ M cAMP and immediately, centrifuge at $20^\circ C$ for 5 min at $1,500 \times g$.
2. Resuspend the cell pellet at 1×10^7 cells/ml in ice-cold GSK3 lysis buffer.
3. Centrifuge lysates for 2 min at $10,000 \times g$ and collect the supernatant.
4. To 5 μ l of each extract add 20 μ l of GSK3 reaction assay buffer containing 20 mg of GSK3 peptide substrate. Include parallel, control reactions that additionally contain 50 μ M LiCl, a GSK3 inhibitor.
5. Incubate reactions at $20^\circ C$ for 8 min.

6. Terminate reactions with 20 μ l 15 mM of phosphoric acid.
7. Spot reactions onto P81 phosphocellulose paper and wash with 7.5 mM phosphoric acid.
8. A comparison of bound radioactivity from reactions incubated with or with LiCl will indicate relative GSK3 activities.
9. Maximum levels of GSK3 activity are observed at 15 min, with decreasing levels observed as the pathway adapts.

**3.12. cAMP Stimulation
of ERK2
Phosphorylation**

1. Remove 70 μ l of cells of cAMP-stimulated cells, as per **Sub-heading 3.9**, and add directly to 30 μ l of protein gel-loading buffer; incubate at 95°C for 10 min. Prestimulus (0 time) control cells are taken, as well as, cells at 15–30 s intervals following simulation with 10 μ M cAMP
2. Load 10 μ l samples onto 4–12% Bis-Tris Gels and electrophorese. Run duplicate gels.
3. Transfer gels to separate nitrocellulose membranes and pre-block in 5% BSA.
4. Immunoblot each separate membrane using α -pT202/pT204-ERK or α -actin. Actin serves as a loading control and runs with a similar mobility to ERK2.
5. ERK2 activation is measured by an increase in levels of pERK2 (*See Note 3*).

**3.13. Folic Acid
Stimulation of ERK2
Phosphorylation**

1. Grow cells to $\sim 2\text{--}5 \times 10^6$ cells/ml at 20°C.
2. Centrifuge cells at 20°C for 5 min at $1,500 \times g$.
3. Wash cells once with PM at 20°C.
4. Resuspend cell pellet in PM at 5×10^7 cells/ml.
5. Shake the cell suspension at 200 rpm for 30 min.
6. Stimulate cells with a 50- μ M final concentration of folic acid. By varying the folate concentration it is possible to determine a dose response curve. Take samples at 0, 15, 30, 45, 60, and 180 s.
7. Remove 70 μ l of cells and add directly to 30 μ l of SDS Loading Buffer; incubate at 95°C for 10 min.
8. Load 10 μ l samples onto 4–12% Bis-Tris gels and electrophorese. Run duplicate gels.
9. Transfer gels to separate nitrocellulose membranes and pre-block in 5% BSA.
10. Immunoblot each separate membrane using α -pT202/pT204-ERK or α -actin. Actin serves as a loading control and runs with a similar mobility to ERK2.
11. ERK2 activation is measured by an increase in levels of pERK2.

4. Notes

1. Developing cells in shaking culture with cAMP bypasses the requirement of cells to produce cAMP endogenously. This allows the study of many mutant cell types. Of course all assays shown can also utilize cells that are allowed undergo normal development.
2. Two methods for determining cAMP binding are presented. One uses PB and is the more physiologic. The second uses saturated $(\text{NH}_4)_2\text{SO}_4$, which detects all surface binding sites, artificially stabilizes cAMP–receptor interactions, and allows for high affinity binding irrespective of receptor interaction with G proteins. More receptor numbers are calculated using the $(\text{NH}_4)_2\text{SO}_4$ assay. In addition, relative binding affinities are increased at least tenfold using the $(\text{NH}_4)_2\text{SO}_4$ assay. For CAR2 and CAR4, it is essential to use the $(\text{NH}_4)_2\text{SO}_4$ assay; in PB, the very low affinities of CAR2 and CAR4 ($>1\ \mu\text{M}$) are masked by the high nonspecific background binding observed when using extremely elevated concentrations of $[^3\text{H}]$ -cAMP. In PB, CAR1, and CAR2 affinities for cAMP are below $1\ \mu\text{M}$.
3. It has been argued that ERK2 undergoes adaptive regulation in response to a continuous saturating dose of cAMP. However, ERK2 regulation is more complex. While the kinase that activates (phosphorylates) ERK2 may adapt, the phosphatase that deactivates (dephosphorylates) ERK2 is inhibited by cAMP and remains inactive in the presence of saturating cAMP (23).

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Chapter 19

Quantifying In Vivo Phosphoinositide Turnover in Chemotactically Competent *Dictyostelium* Cells

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Summary

Phosphoinositide (PI) signalling is one of multiple signalling cascades involved in chemotaxis in *Dictyostelium discoideum*. PI signalling comprises a complex interaction of multiple enzymes, each with multiple phospholipid substrates and thus products, often relying upon several enzymes in series to produce a signal. PI turnover, controlled by both kinases and phosphatases, is also rapidly triggered and spatially constricted. This complexity makes understanding acute regulation of these signalling components problematic. However, the ubiquitous and extensive roles of phospholipids, including phosphatidylinositol-4,5-diphosphate (PI(4,5)P₂), in cell signalling and developmental processes make understanding the production of these compounds of great importance. We have shown the acute reduction of PI phosphorylation in response to a widely used bipolar disorder and epilepsy treatment, valproic acid, as a potential therapeutic role for the drug using chemotactically competent *Dictyostelium*. Here we describe a means for measuring acute in vivo phospholipid labelling in *Dictyostelium*.

Key words: *Dictyostelium*, Epilepsy, Phosphatidylinositol, Phospholipid kinase, Valproic acid

1. Introduction

Following cell stimulation, the transient production of phosphoinositide (PI) compounds by lipid kinases or dephosphorylation by phosphatases at the cell membrane provides a critical link between extracellular signalling components and intracellular effectors (1). Production of PI compounds like phosphatidylinositol-4,5-diphosphate (PI(4,5)P₂) leads to the recruitment of PH domain containing proteins such as actin-binding proteins (ABP) and thereby regulating their activity (2), generally leading to the

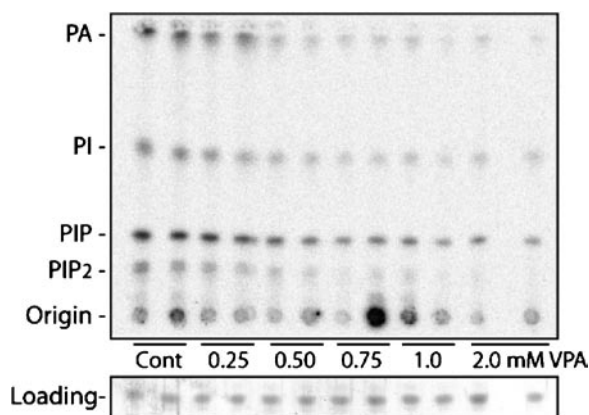


Fig. 1. TLC separation of ^{32}P -labelled phosphoinositides from chemotactically competent *Dictyostelium* cells acutely treated with increasing concentrations of valproic acid. PIP_2 , PIP, and phosphatidic acid (PA) are labelled, as defined by R_f values (0.29, 0.33, 0.82, respectively) (12). Following exposure of the TLC plate to a phosphorimager screen (*top panel*) for quantitative analysis, the TLC plate was stained for total lipids as a loading control (*bottom panel*).

promotion of actin assembly (3). PIP_2 also plays an important role in regulating ion channels (4), in addition to providing a substrate for the production of PIP_3 .

Valproic acid, 2-propylpentanoic acid, was accidentally found to control induced seizures over 40 years ago (5), but its therapeutic target remains unclear. Studies into the mechanism of action of the drug have identified numerous cellular effects both in mammalian models and in *Dictyostelium* (6). These common targets include inositol trisphosphate signalling (7, 8) and MAP kinase activation (9, 10). Our recent studies analysing the effect of valproic acid on chemotaxing *Dictyostelium* cells discovered a novel effect of the drug in reducing PI phosphorylation (11; and Fig. 1), an effect that may provide a new insight into the therapeutic action of the drug.

2. Materials

2.1. Equipment

For efficient implementation of the following protocol, a number of specialist pieces of equipment are recommended, thus have been listed here as suggestion – other branded products may be substituted.

1. Small volume pulser, e.g. Watson Marlow 505Di, to prepare chemotactically competent *Dictyostelium* cells.
2. Vacuum centrifuge (e.g. Genevac personal evaporator) to dry down PI samples (see Note 1).

3. Phosphorimager (e.g. Typhoon Scanner; GE Healthcare), phosphor screen, and cassette (e.g. Fujifilm BAS-MS2025) to quantify radioactive-label incorporation. A light box is necessary for erasing these screens (*see* [Note 2](#)).

2.2. Cell Culturing and Preparation

1. *Dictyostelium* strains are grown in shaking liquid culture using HL5 medium (Formedium) in conical flasks with aeration (120 rpm, 21°C). Liquid medium is supplemented with penicillin and streptomycin (10× concentrated; PAA Laboratories) for wild-type strains, with additional Blasticidin (10 µg/ml; PAA Laboratories) or Geneticin (10 µg/ml; Invitrogen) for transformed strains requiring selection.
2. KK2: 20 mM potassium phosphate buffer, pH 6.1.
3. cAMP (Sigma), 200 mM stock solution (adjust to pH 6.3 with NaOH), store in aliquots at -20°C.
4. Cells can be plated in either 3.5-cm-diameter tissue culture dishes or six-well tissue culture plates (Sarstedt).
5. Microscope for visualising cell adhesion in tissue culture plates.

2.3. Labelling and Isolation of Phospholipids

1. Phosphatase inhibitor cocktails 1 and 2 (Sigma). Store in aliquots at -20°C.
2. [γ -³²P]ATP, 3,000 Ci/mmol (10 mCi/ml) (Perkin Elmer).
3. 2% (w/v) saponin (Sigma) in KK2. Store in aliquots at -20°C.
4. Internal buffer master mix (1.1 ml per well): 139 mM sodium glutamate, 5 mM glucose, 5 mM EGTA, 20 mM PIPES of pH 6.6, 1 mM magnesium sulphate. Just prior to use, add 1/200 volume of each phosphatase inhibitor cocktail, 1/70 volume of 2% saponin, and ~5 µCi [γ -³²P]ATP per ml (*see* [Note 3](#)).
5. Acidified methanol: analytical-grade methanol supplemented with 1/100 volume of concentrated HCl (VWR).
6. Chloroform.
7. Cell Scraper (Fisher Scientific).
8. 2-ml Eppendorf tubes.
9. Suction device for removing radioactive internal buffer (*see* [Note 4](#)).
10. Bench-top centrifuge dedicated to radioactive work.

2.4. Separation of Phospholipids via Thin-Layer Chromatography (TLC)

1. Silica gel 60 TLC plates (VWR): 20 × 20 cm or 10 × 20 cm depending on the number of samples (*see* [Note 5](#)).
2. 2% (w/v) sodium oxalate (Sigma) solution to prime the TLC plate. Dissolve 2 g of sodium oxalate in 80 ml distilled water and then add 20 ml methanol.

3. TLC solvent: chloroform:acetone:methanol:acetic acid:distilled water (40:15:13:12:7). Use analytical-grade solvents (*see* [Note 6](#)).
4. 90°C oven.
5. TLC tank (Camag twin trough chamber, VWR).
6. 3-MM paper (Fisher Scientific).

2.5. Staining Total Lipids

1. Staining solution: 0.5 M copper sulphate and 8.5% phosphoric acid (from 85% stock) in distilled water, store at room temperature. This solution can be reused.
2. 160°C oven.

3. Methods

Cell permeabilization relies upon saponin, a mixture of naturally occurring amphipathic glycosides that forms pores in cell membranes. Incubating cells in the presence of saponin therefore allows rapid entry of labelling compounds into the cell cytosol, and the subsequent *in vivo* labelling of target compounds. The method outlined here has been tailored to provide increased access of [γ - ^{32} P]ATP to *Dictyostelium* cells whilst not causing cell lysis. A role of phosphatases in these experiments is removed by the inclusion of phosphatase inhibitors in the labelling solution (internal buffer). Potential loss of dislodged cells from the culture dish during drug treatment and radio-labelling is also monitored by staining for total lipids. Although the experiment is designed to analyse PI turnover in cells following 5 h of development, the method may be applied to growing cells or to cells at different stages of development.

3.1. Cell Preparation and Labelling of Phospholipids

1. Use cells growing in log-phase ($1\text{--}3 \times 10^6$ cells/ml), harvest 5×10^5 cells for each sample/well by centrifuging ($1,000 \times g$, 5 min), and washing the pellet twice with KK2.
2. Re-suspend the cell pellet in KK2 to give a density of 1×10^7 cells/ml in KK2.
3. Pulse the cells every 6 min for 5 h with cAMP to give a final concentration of 25 nM cAMP per pulse with aeration by shaking (120 rpm) (*see* [Note 7](#)).
4. Pellet cells ($1,000 \times g$, 5 min), wash the pellet twice with KK2 and re-suspend in KK2 to give 3.3×10^5 cells/ml.
5. Apply 1.5-ml cell suspension to each well (5×10^5 cells/well). Incubate plates (21°C, 10 min, *see* [Note 8](#)) to allow the cells attach to the culture dish before checking adhesion using a microscope (*see* [Note 9](#)).

6. If drug pre-treatment is required, remove KK2 and replace by gentle addition of the drug in KK2, and leave for required time period.
7. Remove buffer with suction device (*see Note 10*), and gently replace with 1 ml of radioactive internal buffer to each well. Stagger your time intervals of buffer removal/internal buffer addition.
8. After exactly 6 min for each well, remove the internal buffer using a suction device and immediately add 500 μ l acidified methanol to lyse the cells.
9. When all cells have been lysed, scrape each well with a cell scraper and transfer the lysate from each well to 2-ml Eppendorf tubes.
10. Rinse and scrape each well with an additional 250 μ l of acidified methanol and transfer the lysate to the corresponding Eppendorf tubes.
11. Add 800 μ l of chloroform and 400 μ l water to each Eppendorf and vortex briefly.
12. Centrifuge Eppendorf tubes for 1 min at 1,200 g in a bench-top centrifuge to separate the upper (aqueous) phase and the lower (organic) phase. Transfer the lower (radio-labelled phospholipid-containing) phase to a fresh Eppendorf tube.
13. Dry down the samples in a concentrator or evaporator.

3.2. Separation of Phospholipids via TLC

3.2.1. Preparation of TLC Plates

1. Lightly mark a line approximately 1.5 cm from one edge of the TLC plate with a soft graphite pencil and mark where each sample will be loaded onto the plate at least 1 cm apart (*see Note 11*).
2. Rapidly dip the plate into the 2% sodium oxalate solution and air dry.
3. Incubate the plate in a 90°C oven to reactivate silica (minimum 30 min).
4. Re-suspend each sample in 30 μ l of chloroform immediately prior to loading on the TLC plate.
5. Apply each sample slowly onto loading marks of the still-warm TLC plate to avoid diffusion of the sample onto a large area.
6. Rinse each Eppendorf tube with additional 20 μ l of chloroform, ensuring the chloroform is washed up and down the sides of each tube. Load each sample on the equivalent spot on the TLC plate. Allow to air dry.

3.2.2. Preparation of TLC Tank

1. Work in a fume hood since the solvents are volatile.
2. Mix the solvents (*see Subheading 2.4, item 3*) by swirling in a bottle to avoid aeration prior to adding to the TLC tank.

3. Add the solvent to the bottom of the TLC tank 30 min prior to adding the TLC plate and replace the lid sealed with vacuum grease. It may be necessary to include a sheet of 3-MM paper surrounding the plate on the inner walls of the tank to produce a consistent internal vapour pressure.
4. The required total volume of solvent depends on the tank size. The solvent should not touch the sample spot when the TLC plate is placed into the tank. For a twin trough chamber tank use 100 ml solvent for both chambers.

3.2.3. TLC Separation and Quantification

1. Place the TLC plate in the tank and immediately replace the lid to sustain the vapour pressure.
2. When the solvent front appears approximately in the middle of the plate, remove the plate from the tank (*see* [Note 12](#)).
3. Dry the plate in a fume hood (moisture may damage some screens).
4. Erase the phosphor screen prior to use by exposure on a light box (minimum 30 min).
5. Place the dry TLC plate on the phosphor screen in a cassette and leave for 48 h (dependent upon the amount of radiolabel incorporation).
6. Read the phosphor screen in a phosphorimager and analyse the band intensity with “ImageQuant” software (*see* [Fig. 1](#)) (*see* [Note 13](#)).

3.3. Staining Total Lipids

1. Preheat an oven to 160°C.
2. Rapidly dip the TLC plate in the copper sulphate staining solution and remove excess liquid.
3. Incubate the TLC plate for 20 min at 160°C (*see* [Note 14](#)).
4. Scan or photograph the plate to compare total lipid levels between treatments as a loading control (*see* [Fig. 1](#)).

4. Notes

1. A water pump-based evaporator or a vacuum centrifuge can be used as alternatives.
2. Traditional autoradiography film can be used for recording radiolabel incorporation, although this does not provide linear measurement of radiolabel intensity.

3. Saponin concentration has been defined for use with 5 h developed cells. Using cells starved for longer or shorter periods (e.g. growing cells) may require testing alternative conditions.
4. A suction device, dedicated to this radioactive work, provides a faster and more reproducible removal of labelling buffer in comparison to repeated pipette use.
5. Store silica plates in an airtight container to avoid inhalation of silica powder.
6. TLC solvent constituents and ratios can be varied to alter separation conditions. For example, using chloroform:methanol:water 65:25:4, as solvent separates phospholipids and hexane:diethylether:acetic acid 70:30:1 separates neutral lipids.
7. For pulsing cells, use a flask four times the volume of your cell suspension to ensure proper aeration.
8. Ensure all the following incubation steps and solutions are at 21°C.
9. Cells should be of sufficient density to form an even monolayer in each well.
10. It might be necessary to take samples of, e.g. the total amount of radioactivity used and liquid or solid waste. Take them at appropriate times to complete your radioactivity records.
11. Leave an increased distance between the last two sample marks to identify orientation of loading.
12. An increased “smiling” effect will occur the further the samples separate up the plate.
13. Phospholipids are distinguished by R_f values (for each band, the ratio of distance from the origin to the band over the distance from the origin to the solvent front). Under conditions described here R_f values are: 0.29 (PIP₂), 0.33 (PIP), 0.50 (PI), and 0.82 (phosphatidic acid (PA) [\(12\)](#)). These values are dependent on solvent composition.
14. During copper sulphate staining it is essential to evenly heat the TLC plate during oven baking; therefore, place the plate at the back of the oven parallel to the door.

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Chapter 20

In Vivo Measurements of Cytosolic Calcium in *Dictyostelium discoideum*

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Summary

The involvement of calcium signalling during chemotaxis in *Dictyostelium discoideum* is well documented. Spatiotemporal increases of intracellular calcium ($[Ca^{2+}]_i$) have been observed within seconds of stimulation with the chemoattractants folic acid and cAMP. This rise in $[Ca^{2+}]_i$ localises to the rear cortex of the cell (J. Cell Sci. 109:2673–2678, 1996) and has been found to be not essential for chemotaxis, but likely to be involved in fine tuning of chemotactic responses (EMBO J. 19:4846–4854, 2000). Measurements of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) responses have involved the use of different Ca^{2+} probes including ectopically expressed aequorin (a Ca^{2+} -sensitive photoprotein), the fluorescent dye fura-2-dextran and the radioisotope $^{45}Ca^{2+}$. The aequorin method (J. Cell Sci. 110:2845–2853, 1997) offers nonperturbing, real-time measurement of cytosolic free Ca^{2+} in suspensions of cells, but the low levels of light emission preclude measurements on individual cells. Fura-2 imaging (Cell Calcium 22:65–74, 1997; Eur. J. Cell Biol. 58:172–181, 1992; Biochem. J. 332:541–548, 1998; BMC Cell Biol. 6:13, 2005) has the advantage of allowing Ca^{2+} responses to be observed in individual cells so that the subcellular localisation of the response and differences amongst individual cells can be observed. However data collection is more labour intensive, much smaller numbers of cells are sampled, the cells are unavoidably damaged physically during loading and the time resolution (s) is much less than that provided by the aequorin method (ms). $^{45}Ca^{2+}$ uptake assays (Cell Biol. Int. Rep. 2:71–79, 1978; J. Cell Biol. 112:103–110, 1991) allow measurement of Ca^{2+} influx from the medium by cell suspensions with a time resolution of the order of seconds. Radioactive Ca^{2+} uptake measurements are unsullied by but equally do not provide information about Ca^{2+} efflux, intracellular Ca^{2+} release or sequestration or changes in cytosolic free Ca^{2+} levels.

Key words: *Dictyostelium*, Calcium, cAMP, Folic acid, Aequorin, $^{45}Ca^{2+}$, Fura-2-dextran

1. Introduction

The eukaryotic cellular slime mould *Dictyostelium discoideum* is a widely used model organism as its lifecycle involves both unicellular and multicellular stages. This has made *D. discoideum* an excellent

model for studying the molecular basis of processes such as chemotaxis and cell signalling. During the *D. discoideum* lifecycle, vegetative cells are chemotactically responsive to folic acid, which is secreted by the bacteria they hunt as a food source. During times of nutrient depletion when cells begin to starve, a developmental program regulated by a receptor-mediated signalling system is initiated. Amoebae begin to periodically secrete the chemical cAMP and simultaneously become chemotactically responsive to the same chemical. The result of this secretion and sensitivity to cAMP is the accumulation of $\sim 10^5$ amoebae at an aggregation centre. The aggregated cells then develop from individual amoebae into a multicellular stage called a slug. During this period, cells differentiate into prespore and prestalk cells, and subsequently culminate to form a fruiting body consisting of a droplet of dormant spore cells resting atop a stalk. Studying the processes by which individual amoebae are able to signal to each other during aggregation and differentiation has elucidated numerous components involved in chemotaxis signalling pathways.

Intracellular signals that are responsible for chemotaxis are activated by binding of cAMP and folic acid at concentrations in the nanomolar range, to specific cell surface G-protein coupled receptors (GPCR). Amongst the intracellular signals that are activated by these GPCRs is a transient increase in cytosolic Ca^{2+} concentrations that occurs mainly in the rear of the cell where it fine-tunes chemotactic behaviour (1, 2). Ca^{2+} responses have been measured using recombinant aequorin (3), the fluorescent dye fura-2 (4, 5, 6, 7) or radiolabelled $^{45}\text{Ca}^{2+}$ (8,9). cAR-1, the receptor activated by cAMP (10), and an unidentified GPCR receptor that binds folic acid, also rapidly activate phospholipase C and guanylate cyclase via a G-protein-dependent pathway. This results in increased intracellular inositol-1, 4, 5-trisphosphate (IP3) and guanosine 3',5'-cyclic monophosphate (cGMP) concentrations within seconds (11, 12, 13).

The transient accumulation of Ca^{2+} in the cytosol after a chemoattractant stimulus is due to influx from both the extracellular medium (9, 14, 15) and intracellular stores (16, 17). The kinetics of these Ca^{2+} responses to both folic acid and cAMP have been characterised. Basal $[\text{Ca}^{2+}]_c$ ranges from ~ 50 to 90 nM and Ca^{2+} entry into the cytosol begins 7–15 s after chemoattractant stimulation. The response is transient, so that $[\text{Ca}^{2+}]_c$ peaks 20–30 s after stimulation, then declines due to sequestration by Ca^{2+} -ATPases (18, 19), returning to basal levels about 40–60s post stimulation (3). Average peak Ca^{2+} responses to cAMP in developed cells are higher (~ 200 nM) than the responses to folic acid in vegetative cells (~ 170 nM). The influx of Ca^{2+} via cAR1 activation has been shown to be partly mediated by the G-protein $\text{G}\alpha 2\beta\gamma$ during early development, and independent of $\text{G}\alpha 2\beta\gamma$ during the later stages of development (20). However the pathway that mediates the folic acid response has been shown to act solely via

the G-protein $G\alpha 4\beta\gamma$, as $G\alpha 4$ and $G\beta$ null mutants lack stimulated Ca^{2+} influx (21).

Measurements of $[Ca^{2+}]_i$ in whole cells have been integral in studying the temporal and local changes that occur during chemotactic responses. Various methods of measuring $[Ca^{2+}]_i$ in *D. discoideum* have provided evidence for this transient increase. These include loading cells with the fluorescent Ca^{2+} indicator fura-2-dextran and imaging individual cells, measuring Ca^{2+} uptake using the radioisotope $^{45}Ca^{2+}$, and in vivo expression of the Ca^{2+} -sensitive photoprotein, aequorin.

Abe et al. (22) were the first to provide direct evidence of Ca^{2+} fluxes in response to chemoattractants, after using electroporation to load *D. discoideum* cells with the fluorescent Ca^{2+} indicator, fura-2. Later it was found that this method of introduction of the dye caused damage to the cells if the condenser was charged at too high a voltage, in that they became rounded and unable to adhere to substratum. This was overcome by pulsing the cells at 850 V, as too low a voltage did not allow cells to take up the dye (4). Furthermore, Schlatterer et al. (5) reported that the indicator was rapidly taken up into intracellular vesicles. To overcome this, fura-2 was covalently linked to dextran to prevent sequestration. Contrary to reports by Yumura et al. (23), Sonnemann et al. (4) found when using electroporation, that the amount of indicator taken up was lower than for cells loaded by the scrape loading method. Scrape loading involves tightly attaching cells to coverslips and scraping them with a rubber policeman to tear the cell membrane (5). This introduces macromolecules into the cell and the cell membranes are immediately repaired.

Sonnemann et al. (4) used both scrape loading and electroporation to successfully load cells with fura-2-dextran and measure $[Ca^{2+}]_i$ changes in response to 0.1–1 μM cAMP. The results were in conflict to findings reported previously by Schlatterer et al. (5, 24), who scrape loaded cells with fura-2-dextran and reported no detectable change in $[Ca^{2+}]_i$ after stimulation with submicromolar concentrations of cAMP. These contradictions may reflect the technical difficulties which are encountered when loading *Dictyostelium* cells with fluorescent Ca^{2+} -sensitive indicators using either of these two methods.

An alternate method of measuring Ca^{2+} responses to chemoattractant in intact cells is to measure the uptake of the radioisotope $^{45}Ca^{2+}$. Both the strength and limitation of this method is that it allows only measurements of Ca^{2+} entry into the cell. Samples must be taken at regular intervals over the course of the response and individually counted using a liquid scintillation counter. Milne and Coukell (9) developed a $^{45}Ca^{2+}$ uptake assay to successfully measure Ca^{2+} accumulation in *D. discoideum* in response to both cAMP and folic acid. The Ca^{2+} responses reported were consistent with those later reported by Sonnemann et al. (4).

In an attempt to overcome the problems associated with measurements using both fura-2-dextran and $^{45}Ca^{2+}$, a non-perturbing

method to measure $[Ca^{2+}]_c$ was developed. Saran et al. (25) expressed the Ca^{2+} -sensitive recombinant photoprotein, aequorin, in *D. discoideum*. However, the relatively low Ca^{2+} affinity of native aequorin impeded the measurements of the nanomolar fluxes in $[Ca^{2+}]_i$ in response to chemotactic stimulation. Nebl and Fisher (3) independently used a semisynthetic aequorin, which exhibits improved sensitivity to Ca^{2+} and light emission properties (26). This more sensitive form of aequorin allowed the first accurate, real-time measurements of basal $[Ca^{2+}]_c$ and transients during chemotactic responses. These authors reported that the responses elicited by cAMP and folic acid are dose dependent and saturate between 4 and 20 μ M. The study confirmed that the responses are dependent on the presence of free extracellular Ca^{2+} as reported by Milne and Coukell (9).

2. Materials

2.1. Aequorin Method

To enable in vivo cytosolic Ca^{2+} measurements, the cells must express the recombinant Ca^{2+} -sensitive photoprotein, apoequorin. To enable this, an expression vector was constructed by insertion of the 0.6-kb coding region of apoequorin cDNA, clone pAQ2 (27) into the *Dictyostelium* plasmid pDNeo2. The resulting construct, pPROF120, contains a gene fusion between the first eight codons of the *Dictyostelium* actin 6 gene and the apoequorin gene. Expression and termination of the actin 6-apoequorin fusion protein are regulated by the *Dictyostelium* actin 6 promoter and actin 8 transcription terminator, respectively (3).

To obtain aequorin-expressing derivatives of the strains of interest they are transformed with the plasmid pPROF120 (3), for example by using either the calcium phosphate coprecipitation method (28) or electroporation (29) and G418 selection in liquid medium (HL-5) or on lawns of *Micrococcus luteus* (30). The resulting transformants are used to analyse $[Ca^{2+}]_c$ in both resting and chemotactically stimulated wild-type cells.

The protein produced by transformants expressing apoequorin cDNA lacks its prosthetic group, coelenterazine, without which it is totally inactive. In vivo reconstitution of the functional photoprotein is achieved by incubating cells in growth medium or development buffer containing the chromophore and the dispersing agent, Pluronic F-127, which enhanced reconstitution and cell loading. As the chromophore is highly hydrophobic, the use of a methanolic stock solution containing Pluronic-F127 also significantly enhances cell loading (3).

2.1.1. Cell Culture and Loading

All buffers should be prepared with milliQ deionised water which has been treated with activated charcoal. Place ~ two teaspoons of charcoal in 1 L of milliQ water and allow to settle at 4°C overnight; then filter through Whatman 3-mm chromatography paper (Whatman Schleicher & Schuell) and sterilise.

1. HL5 medium: 0.43 g/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.35 g/L KH_2PO_4 , 10 g/L glucose, 10 g/L proteose peptone (Bacto), 5 g/L yeast extract (Bacto), pH 6.4.
2. Coelenterazine-*h* (Molecular Probes): Coelenterazine-*h* is light- and oxygen-sensitive and is delivered, sealed under an argon atmosphere. Prepare 10- μg aliquots by dissolving 300 μg of coelenterazine in 300 μL methanol. Dispense in 10- μL aliquots into microcentrifuge tubes, dry quickly under vacuum, and store protected from light at $\leq -20^\circ\text{C}$.
3. 20% (w/v) Pluronic-F127 (Sigma) dissolved in methanol.
4. MES development buffer (MES-DB): 10 mM MES (sodium salt, Sigma) of pH 6.2, 10 mM KCl, 0.25 mM CaCl_2 .

2.1.2. Calcium Measurements

1. Lysis Buffer: 10 mM MOPS (Sigma) of pH 7.2, 10 mM Ca^{2+} -acetate, 1% (v/v) Triton-X100 (Sigma).
2. Stock solutions of chemoattractants in deionised water: 20 mM cAMP (Boehringer Mannheim, GmbH); 20 mM folic acid.

2.2. Fura-2-Dextran Loading Method

1. Sørensen's Phosphate Buffer (SP buffer): 17 mM ($\text{KH}_2/\text{Na}_2\text{H}$)- PO_4 , pH 6.0.
2. Poly-L-lysine, 10 $\mu\text{g}/\text{mL}$ (Sigma).
3. H5-buffer: 5 mM Hepes, 5 mM KCl, pH 7.0.
4. H50-buffer: 20 mM Hepes, 50 mM KCl, 10 mM NaCl, 1 mM MgSO_4 , 5 mM NaHCO_3 , 1 mM NaH_2PO_4 , pH 7.0.
5. Fura-2-dextran (Mobi Tec, Göttingen, Germany).
6. Chemoattractant stock solution: 100 μM cAMP.
7. Calibration buffer: 10 mM Pipes, 5 mM NaCl, 50 mM KCl, 10 mM EGTA (dipotassium salt), pH 7.0.

2.3. $^{45}\text{Ca}^{2+}$ Method

2.3.1. Cell Culture and Loading

1. HL5 medium: 0.43 g/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.35 g/L KH_2PO_4 , 10 g/L glucose, 10 g/L proteose peptone (Bacto), 5 g/L yeast extract (Bacto), pH 6.4.
2. $^{45}\text{CaCl}_2$ (544 MBq/mg Ca^{2+}) (ICN, Biomedicals, St. Laurent, Canada).
3. Development buffer: 10 mM Na_2HPO_4 , 1 mM MgCl_2 , 0.2 mM CaCl_2 , pH 6.2.
4. Non-nutrient agar: 1–1.5% agar.
5. H buffer: 20 mM Hepes/KOH, 5 mM KCl, pH 7.0.

6. Uptake medium: 20 mM Hepes-KOH, 5 mM KCl, 100 μ M CaCl_2 , $\sim 0.5 \mu\text{Ci } ^{45}\text{CaCl}_2$, pH 7.0.
7. Stock solutions of chemoattractants in deionised water: 20 mM cAMP; 20 mM folic acid.
8. NCS tissue solubiliser, PCS scintillant (Amersham Corp., Oakville, Canada).

3. Methods

3.1. Aequorin Method

3.1.1. Cell Culture and Loading

1. Grow cells in ~ 100 mL HL5 medium at 21°C with aeration on an orbital shaker at 150 rpm, to a density of $2\text{--}3 \times 10^6$ cells/mL for developmental assays, or 1×10^6 cells/mL for vegetative assays (*see Note 1*).
2. Harvest 1×10^8 cells by centrifugation at $1,000 \times g$ for 2 min. Wash the pellet by resuspending in 20 mL MES-DB and recentrifuge. Repeat twice for developmental assays (*see Note 2*).
3. Resuspend the pellet in 5 mL (2×10^7 cells/mL) of MES-DB for developmental assays (cAMP stimulation), or 5 mL HL5 medium for vegetative assays (folic acid stimulation) (*see Note 3*).
4. Prepare coelenterazine-*h* by dissolving one 10- μ g aliquot in 20 μ L of 20% (w/v) Pluronic-F127 and, once it is dissolved, adding 200 μ L dH_2O . 55 μ L of this preparation is sufficient to treat 1×10^8 cells to give a final concentration of 0.5 $\mu\text{g/mL}$ coelenterazine-*h*.
5. Incubate the samples with aeration by shaking at 120 rpm at 21°C . For experiments using vegetative cells, incubate 4–5 h to allow the cells to load properly with coelenterazine-*h* and allow reconstitution in vivo with aequorin. For assays with developed cells incubate until the desired stage of development has been reached (6–8 h for maximal responsiveness to cAMP) (*see Note 4*).
6. After incubation, pellet the cells by centrifugation (as earlier), wash once with 20 mL MES-DB to remove excess coelenterazine-*h* and resuspend the pellet in 5 mL MES-DB ready for assay.

3.1.2. Measurement of Total Light Emission: The Lysis Step

The total light emission possible from each sample of cells must be measured prior to the $[\text{Ca}^{2+}]_c$ assay. This allows normalisation of the signals recorded from the cells so that $[\text{Ca}^{2+}]$ can be calculated using the in vitro concentration-effect curve of aequorin luminescence. This is done as follows with our experimental setup with a New Brunswick ATP Photometer (**Fig. 1**) (*see Notes 5 and 6*).

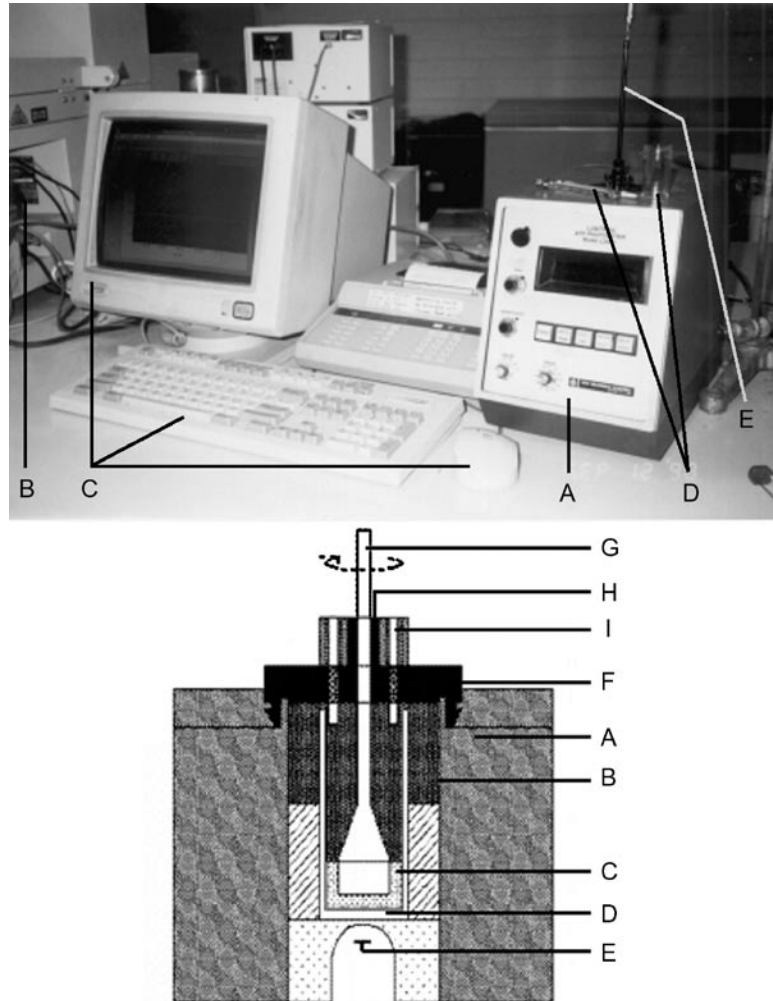


Fig. 1. Apparatus for assay of aequorin. *Upper panel:* (A) New Brunswick Scientific Lumitran® model L-3000 photoluminometer unit housing the photomultiplier, all the associated electronics and manual controls; (B) model PCI-20428 multifunction I/O data acquisition card; (C) computer monitor, keyboard and mouse; (D) Hamilton syringe and assay cuvette; (E) rotor shaft. *Lower panel:* Sectional diagram of the reaction chamber and photomultiplier housing adapted for the purpose of measuring aequorin luminescence in stirred suspensions of stimulated *Dictyostelium* cells. (A) Photometer housing; (B) reaction chamber; (C) cell sample; (D) reaction cuvette; (E) photomultiplier; (F) stainless steel coverslip; (G) rotor shaft; (H) rotor housing; (I) needle guide with rubber septum (Modified from [ref. 34](#)).

1. Prepare serial dilutions of cell preparations to 10^{-2} and 10^{-3} in MES-DB.
2. Place 5 mL lysis buffer into a standard 20-mL standard assay vessel fitted immediately in front of a low-noise photomultiplier. Our setup includes a mechanical stirrer that stirs the cell suspensions at 100 rpm to allow for total lysis or proper distribution of chemoattractant ([Fig. 1](#)).

3. Adjust the background settings in the software so that background counts from the luminometer with the shutter closed are subtracted in real time, bringing the base line to zero (*see Note 7*).
4. Set the software to integrate, open the light shutter, and inject 500 μL of the 10^{-3} cell dilution into the lysis buffer using a syringe. Record the total amount of light discharged; this will then be used during the Ca^{2+} measurements (*see Note 7*).

3.1.3. Calcium Measurements: The Assay Step

1. Prepare a fresh working stock solution of 100 μM folate or 100 μM cAMP in deionised H_2O .
2. Transfer cell suspension to a fresh clean standard assay vessel; place in luminometer and activate the stirrer.
3. Enter the recorded total light emission (recorded from **Subheading 3.1.2**) and the dilution factor (the final dilution is 10^{-4} , as 500 μL of the 10^{-3} dilution of cells in the lysis step, was injected into 5 mL of lysis buffer). Allow the basal Ca^{2+} levels to stabilise; this may take up to 1 min. In wild-type cells the basal Ca^{2+} levels should be between 50 and 100 nM.
4. Stimulate cells by injecting 50 μL of chemoattractant (typically 100 μM to yield 1 μM final concentration) into the cell suspension using a syringe, and record the response. An example of typical responses to 1 μM cAMP and 1 μM folic acid in wild-type cells is shown in **Fig. 2**.

3.1.4. Calculation of $[\text{Ca}^{2+}]_i$ from Light Emission

Correction for aequorin consumption throughout an experiment, as well as normalisation and conversion of *in vivo* signals into values of $[\text{Ca}^{2+}]_i$ was achieved in real time in the computer using our own purpose-designed software written in the graphical programming language (33) that was provided with software for the data logging board. However the same outcome can be achieved by recording luminescence in raw light units and performing the calculations post hoc as follows (34).

1. Calculate for each time point of the initial cell lysis step and the assay step

$$L = R - B$$

where L is the background adjusted luminescence, R is the total raw luminescence, and B is the background luminescence (measured with shutter closed).

2. Calculate for the lysis step

$$T = rnt \sum_{i=1 \rightarrow n} L_{\text{lysis}}$$

where T is the total light emission from aequorin in the undiluted cells in the sample to be assayed, r is the ratio of the total number of cells in the assay to the number of cells in the diluted aliquot

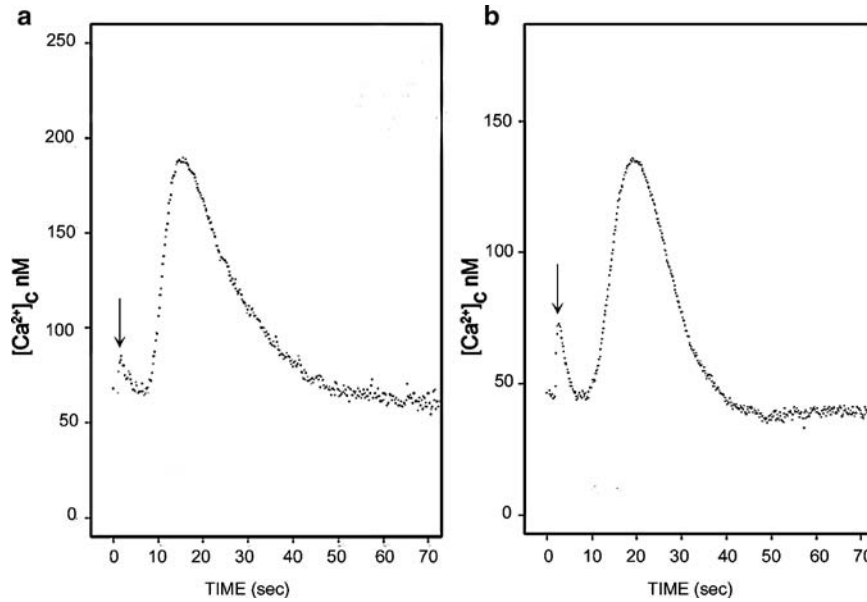


Fig. 2. Cytosolic Ca^{2+} responses recorded in wild-type cells in response to $1 \mu\text{M}$ cAMP (cells at 6 h development) (a) and $1 \mu\text{M}$ folate (vegetative cells) (b). The chemoattractants were delivered within 1 s of the onset of recording as indicated by the arrows. Both folate- and cAMP-induced Ca^{2+} responses are developmentally regulated and exhibit similar kinetics. They are dependent on the relative rather than the absolute magnitude of increases in attractant concentration. Responses began after a short delay of 5–10 s. The $[\text{Ca}^{2+}]_c$ reaches a maximum after ~25 s and then returns to basal level within ~60 s after stimulation.

used in the lysis step, t is the time in seconds between successive measurements, L_{lysis} is the light emission at each measurement time during cell lysis, and n is the number of measurements during cell lysis.

3. Calculate for the assay step

$$F_j = \log_{10}(tL_j/T_j)$$

where F_j is the logarithm of the fraction of aequorin-generated light “consumed” during the measurement at each time point j during the assay, L_j is the light emission at time point j during the assay, and $T_j = T - t \sum_{i=1 \rightarrow j} L_{\text{assay}}$ (i.e. T_r is total light remaining at time j after accounting for light [aequorin] “consumption” during the course of the assay) (see Note 8)

4. Calculate $[\text{Ca}^{2+}]$ for the assay step

$$[\text{Ca}^{2+}]_j = 10^{(9+(F_j-12.41955)/2.9)} \quad \text{for } F_j > -7.03$$

$$[\text{Ca}^{2+}]_j = 10^{(9+(-17.1674+\sqrt{37.03672+4.37004F_j})/2.18502)} \quad \text{for } F_j \leq -7.03$$

where $[\text{Ca}^{2+}]_j$ is the intracellular free $[\text{Ca}^{2+}]$ at time j .

3.2. Fura-2-Dextran Loading Method

The following method is adapted from Schlatterer et al. (5) and Sonnemann et al. (4).

3.2.1. Scrape Loading of Cells with Fura-2 Dextran

1. Grow cultures axenically in HL5 at 21°C, shaking at 150 rpm to $3\text{--}8 \times 10^6$ cells/mL. To induce development, harvest 1×10^8 cells and wash three times with ice-cold SP-buffer, resuspend in SP-buffer at a density of 2×10^7 cells/mL, and shake at 21°C on a rotary shaker at 150 rpm.
2. When cells have reached the desired time of development wash once in H5-buffer, resuspend to a density of 1×10^7 cells/mL and shake in this buffer until use.
3. Coat clean glass microscope slides with poly-L-lysine by pipeting 2 mL of an aqueous 10 µg/mL solution of poly-L-lysine (8–14 kDa) onto the slides. Incubate for 3 h and decant the poly-L-lysine solution.
4. Wash cells in H50-buffer and add 500 µL of the cell suspension at a density of 1×10^7 cells/mL to the coated glass slide. Allow the cells to settle for 25 min. Decant the supernatant and wash any non-adherent cells off the slide by dipping the slide in H50-buffer, and remove any remaining fluid.
5. Pipette 250 µL of 5 mg/mL fura-2-dextran (*see* [Note 9](#)) in H50-buffer onto the slide.
6. Scrape the cells off immediately with a rubber policeman and transfer to a microcentrifuge tube on ice.
7. Remove extracellular dye by washing the cells three times with ice-cold H30-buffer. Wash once with H5-buffer and resuspend in 100 µL immediately before placing 10 µL of cells onto glass coverslips. All measurements should be conducted in this buffer ([5](#)).

3.2.2. Loading with Fura-2-Dextran by Electroporation

1. Harvest cells and suspend in ice-cold SP buffer to a density of 5×10^7 cells/mL with 5 mg/mL fura-2-dextran and 1 mM Ca^{2+} . Place 20 µL of the suspension in a 2-mm electroporation cuvette and pulse once with 850 V at 3 µF and 200 Ω in an electroporator (GenePulser, BioRad). The time constant should be approximately 0.6 ms (*see* [Note 10](#)).
2. Immediately add 80 µL of ice-cold 5 mM MgCl_2 in SP buffer, and wash the cells three times in cold H5-buffer.
3. Resuspend the cells in 100–150 µL of H5-buffer and place 10 µL of the cell suspension on glass coverslips and incubate in a humid chamber until assay ([4](#)).

3.2.3. Stimulation with Chemoattractant

1. 10–15 min prior to experimentation add 90 µL of H5-buffer + 1 mM CaCl_2 to the cells.
2. Transfer the slide to the stage of an inverted microscope.
3. For local stimulation of cells place a glass capillary filled with 10^{-4} M cAMP at a distance of 10–20 µm from the cell.
4. For global stimulation add 10 µL of 10 µM cAMP to the fluid covering the cells ([4](#)).

3.2.4. Microscopy, Image Acquisition and Analysis

1. Examine cells by fluorescence microscopy using a 100× objective and 50-W mercury lamp fitted with rotating 340- and 380-nm excitation filters (the latter paired with a 30% neutral density filter). For each filter use 500-ms excitation residence time in the light path and 126 ms change time between filters (4, 35).
2. Record the fluorescent images using a low-light camera of preferably cooled CCD image intensification camera (*see Note 11*).
3. Digitise the images recorded on the camera using a frame grabber and processor card triggered by the rotating filter wheel so that image acquisition begins as soon as the excitation filter is in position.
4. Store each captured image from the frame grabber on the hard disc of the computer, subtract the background image, calculate and display in pseudocolour the image formed by the ratio (R340/380) of the 340 and 380 nm images at each time point (*see Note 12*).
5. Calculate the $[Ca^{2+}]$ concentration at each pixel within images of individual cells from the fluorescence ratio (R340/380) represented by the grey level in the ratio image.

3.2.5. Fluorescence Ratio: $[Ca^{2+}]$ Calibration

1. Wash glass coverslips with 10 mM EGTA and then twice with double deionised water.
2. Prepare calibration solutions with defined free $[Ca^{2+}]$ by adding aqueous $CaCl_2$ to the calibration buffer and adjusting the pH to 7.0. Use the association constant 4.831×10^6 M for Ca-EGTA at an ionic strength of 100 mM (*see Note 13*).
3. Dilute the 5 mg/mL fura-2-dextran stock solution 100-fold into each calibration solution.
4. Pipette 5 μ L of each solution onto a large glass coverslip and place a smaller coverslip atop to give a fluid thickness of 20 μ m.
5. Record image pairs of each solution, make measurements from the digitised ratio images as described in **Subheading 3.2.4**, and plot the ratios of R 350/380 or R 340/380 against pCa (5). An example is shown in **Fig. 3**.

3.3 $^{45}Ca^{2+}$ Method

The following method is based on that described by Coukell and Cameron (31), Milne and Coukell (9) and Veltman et al. (32).

3.3.1. Cell Culture

1. Grow cells axenically in HL5 medium at 21°C on an orbital shaker (150 rpm) until they reach a density of $2-3 \times 10^6$ cells/mL.
2. Harvest 1×10^8 vegetative amoebae, as described in **Subheading 3.1.1**, either at the vegetative stage or at the desired developmental stage. To obtain aggregation competent cells, harvest vegetative cells, wash three times in development

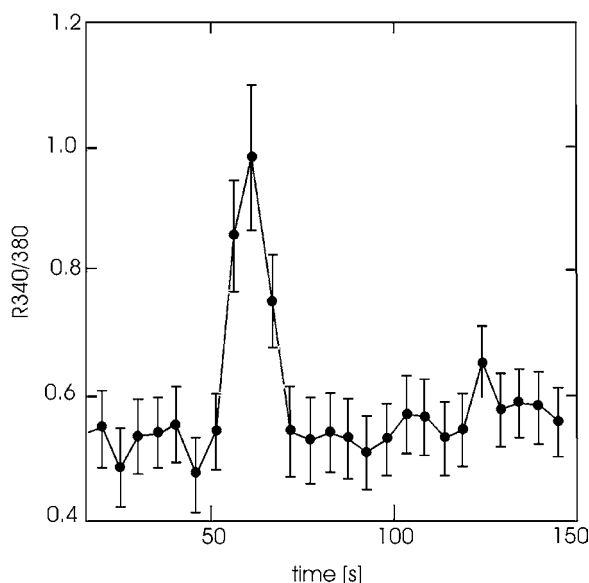


Fig. 3. Cytosolic Ca^{2+} increase induced by cAMP in electroporated cells loaded with fura-2-dextran at 4 h development. Mean $\text{R340/380} \pm \text{SEM}$ of nine cells tested at 6.5 h is plotted versus time; R340/380 was taken as a measure for $[\text{Ca}^{2+}]_i$. The time of addition of cAMP ($1 \mu\text{M}$) was at 45 sec. The experiment was done in H5-buffer containing $1 \text{ mM } \text{Ca}^{2+}$ (Redrawn from [ref. 4](#)).

buffer and spread 1×10^7 cells onto non-nutrient agar. Allow the plates to dry and incubate at 21°C until the cells have reached the desired developmental stage. To harvest the cells, gently suspend them in 10 mL of development buffer with a glass rod, pool 1×10^8 cells into a single centrifuge tube and pellet them by centrifugation (as in [Subheading 3.1.1](#)).

3. After harvesting the cells, wash twice in H buffer, resuspend 1×10^8 cells/mL in H buffer and shake at 21°C and 250 rpm on a gyratory shaker 10 min prior to experimentation.

3.3.2. $^{45}\text{Ca}^{2+}$ Uptake due to Chemoattractant Stimulation

1. Initiate Ca^{2+} uptake by adding 100 μL cell suspension to a microcentrifuge tube containing uptake medium. For measurements of uptake into chemoattractant stimulated cells, supplement the uptake medium with the desired concentration of folate or cAMP. Milne and Coukell ([9](#)) used 40 μM folate and 2 μM cAMP (see [Note 14](#)).
2. Allow uptake to occur for the desired time period. In wild-type cells responses to chemoattractants have usually terminated by ~ 60 s after the addition of chemoattractant (see [Note 15](#)). At each desired time point terminate $^{45}\text{Ca}^{2+}$ entry by addition of 100 μL ice-cold H buffer containing 775 mM CaCl_2 .
3. Pellet each sample of cells by centrifugation for 4 s in a bench top microcentrifuge set at top speed (ca. 14,000 $\times g$) and

discard the supernatant (the centrifuge does not reach final speed in this short time, which is determined by prior testing to be just sufficient to pellet the cells efficiently). Resuspend the pellet in 1 mL of ice-cold H buffer containing 10 mM CaCl_2 , repellet and solubilise the cells in 100 μL NCS tissue solubiliser (31).

3.3.3. Counting

1. Add 1 mL of PCS scintillant to the solubilised pellet and determine the radioactivity using a liquid scintillation counter (31, 32).
2. Normalise the $^{45}\text{Ca}^{2+}$ counts in the cells relative to the maximum observed in the experiment which is taken to be 100%. An example of the effects of cAMP concentration on the magnitude and time course of cAMP mediated Ca^{2+} uptake as determined by Milne and Coukell (9) is shown in Fig. 4.

3.3.4. Non-specific Calcium Uptake

1. To determine the unstimulated rate of Ca^{2+} uptake by resting cells, add the cells to uptake medium containing 225 mM CaCl_2 and process as in Subheading 3.3.2.
2. Normalise the counts as in Subheading 3.3.3 against the same maximum value for the experiment. Determine the chemoattractant induced Ca^{2+} uptake by subtracting the amount of Ca^{2+} taken up by resting cells from the amount accumulated in the stimulated cells (9).

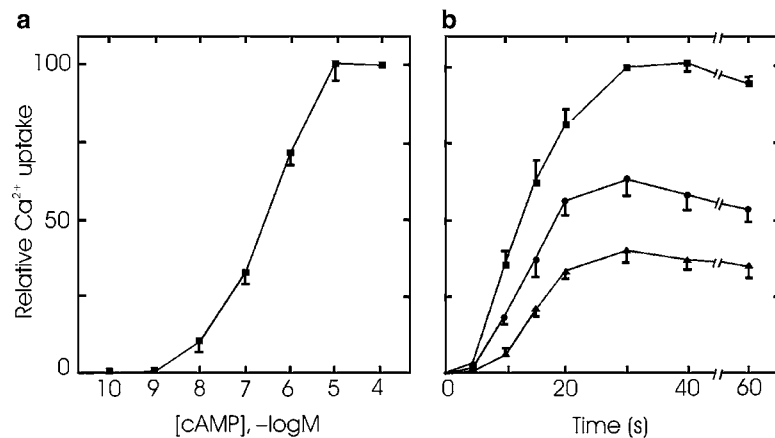


Fig. 4. Effect of cAMP concentration on the magnitude (a) and time course (b) of cAMP-mediated Ca^{2+} uptake measured using $^{45}\text{Ca}^{2+}$. Aggregation competent cells were assayed for cAMP-dependent Ca^{2+} uptake except that in (a) uptake was followed for 30 s in the presence of 0.1 nM, to 100 μM cAMP, and in (b) the assay system contained 100 nM (filled triangle), 1 μM (filled circle), or 100 μM (filled square). cAMP induced Ca^{2+} uptake values are expressed relative to the 30 s time point value in the presence of 100 μM cAMP. Each point is the mean $\pm$ SEM of results obtained in three (a) or four (b) separate experiments (Redrawn from ref. 9).

4. Notes

1. It is important that the density of the cell culture used for folic acid assays is not more than 1×10^6 cells/mL. At higher densities, nutrients may become limiting which could result in decreased responsiveness to folic acid. During early starvation-induced development, responsiveness to folic acid declines rapidly (3).
2. To ensure development initiates properly it is crucial that all nutrient medium is washed properly from the cell pellet and all traces of liquid removed before the final resuspension. For assays with vegetative cells, one wash in MES-DB before resuspension in HL5 is sufficient and cells should be left out of nutrient medium for the minimum amount of time possible to prevent them from beginning to develop.
3. Individual samples can be incubated in 50-mL Falcon tubes; however, when more than one measurement is to be taken from a particular strain, it is preferable to incubate the cells in a larger volume (2×10^7 cells/mL) to ensure even distribution and loading of coelenterazine-*h*, and uniform development. 5-mL aliquots can then be withdrawn just prior to assay.
4. Reconstitution of aequorin increases with time, with 100% reconstitution occurring ~ 6 h after addition. However, reconstitution is sufficient to allow assay at ≥ 1 h after addition.
5. To keep the cells healthy while the light integration step is being conducted, samples should be kept aerated with gentle agitation. Also, as the cells are now suspended in MES-DB, it is important that this step be conducted quickly in the case of folate experiments. The responsiveness of cells to folate begins to diminish over time; therefore, the Ca^{2+} measurements should be taken within ~ 30 min of the cells being removed from HL5.
6. Other luminometers may require slight adjustments in cell suspension and reagent volumes. For example, with a Lumat LB9507 luminometer, it was possible to use ca 3×10^7 cells/mL in a 3-mL suspension with injection of attractants in 300 μL volumes (36).
7. The background counts with the light shutter closed can also be recorded and subtracted post hoc if the luminometer hardware/software makes this necessary. The lysis buffer contains excess Ca^{2+} so that all active aequorin released from lysed cells will be discharged and emit light. The total light emission from this aliquot thus provides a measure of the total active aequorin in the cells being used in the assay. The custom software we wrote uses this information to calculate in real time the

fractional rate of aequorin consumption in the cells and thence the concentration of free Ca^{2+} . The same calculations can also be made post hoc if the raw light emission is recorded.

8. It is assumed in these calculations that the measurement time interval, t , for each time point is constant. If this is not the case then the actual time interval t_j between measurements j and $j-1$, would need to be recorded and used in the calculations. The equations relating $[\text{Ca}^{2+}]$ to the logarithm of the fractional rate of aequorin consumption (F_j) were determined empirically by Nebl and Fisher (3) for aequorin loaded with the cofactor coelenterazine h . The equations would differ for other forms of coelenterazine.
9. To prevent sequestration of the indicator from the cytosol into intracellular vesicles after loading, the fura-2 used should be covalently linked to dextran (commercially available). However, Schlatterer et al. (24) were able to successfully take measurements of cytosolic $[\text{Ca}^{2+}]$ using fura-2 (not dextran linked), provided the measurements were completed within 1 h of loading.
10. With the use of lower voltage pulses, cells do not take up the dye, and conversely, when higher voltages are used, cells become severely damaged and swollen (23). Sonnemann et al. (4) reported electroporation efficiency to be independent to the stage of development of the culture, up to 7 h. However, the amount of fura-2-dextran taken up by individual cells did show some variation. Also, using electroporation to load the cells resulted in lower uptake as compared to scrape loading. The loading efficiency was not able to be increased, even with the use of 2.5 times higher concentration of fura-2-dextran in the electroporation mixture.
11. Schlatterer and Schaloske (35) used a low-light SIT camera (Heimann, Wiesbaden, Germany). However, to increase the sensitivity Sonnemann et al. (4) used an intensified CCD camera (model HLA, Proxitronic, Bensheim, Germany) to capture images of the cells. Use of the CCD camera increases the sensitivity of the image captured by a factor of 10^3 . This allows more accurate measurements of cells that have incorporated low amounts of the indicator therefore are only weakly fluorescent. Levels of less than $10\text{ }\mu\text{M}$ of the indicator were able to be detected (4).
12. Schlatterer and Schaloske (35) used a Data Translation frame grabber/processor model DT 2851/DT 2858 (Bietigheim-Bissingen, Germany) with a resolution of 512×512 pixels and 256 grey levels.
13. Preparing buffers with defined free $[\text{Ca}^{2+}]$ is fraught with difficulty because of unknown, but often significant contributions

of Ca^{2+} from glass surfaces, water supply, reagents, etc. It is easier and more accurate to purchase prepared buffers. Nebl and Fisher (3) and Traynor et al. (2) used the Calcium Calibration Kit I (Molecular Probes).

14. Chemoattractant concentrations may be altered as appropriate for the assay. However, the effects of folate and cAMP on the Ca^{2+} responses are dose dependent and saturated between 4 and 20 mM (3). Milne and Coukell (9) reported half-maximal uptake occurred at ~ 135 nM for folate and at ~ 280 nM for cAMP; however, it must be taken into account that these values are almost double those reported by Nebl and Fisher (3).
15. As this method for measuring cytosolic $[\text{Ca}^{2+}]$ only allows measurements of influxed Ca^{2+} , samples must be taken and read at intervals over the duration of the response. When plotted, this will give an indication of the kinetics of the influx of Ca^{2+} over time.

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Chapter 21

Chemokine Receptor Signaling and HIV Infection

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Summary

The primary function of HIV-1 binding to its chemokine coreceptors is to mediate fusion and viral entry. However, it has been known that this interaction also triggers a variety of signaling cascades. It is likely that the virus-mediated signaling events may facilitate viral infection in various settings where the cellular conditions need to be primed. This has been exemplified recently in our findings that HIV-1 employs envelope-CXCR4 interaction to activate a cellular actin depolymerization factor, cofilin, to support viral latent infection of resting CD4 T cells. Activation of cofilin promotes the cortical actin dynamics that are critical for viral intracellular migration across the static cortical actin barrier in resting T cells.

Key words: HIV-1, CD4 T cell, gp120, CXCR4, F-actin, Cofilin, Phalloidin, Western blot

1. Introduction

HIV-1 infection is initiated when the viral surface glycoprotein gp120 binds to the CD4 receptor (1, 2) and the CCR5 (3) or CXCR4 chemokine coreceptor (4). This interaction leads to viral entry into host cells (5). The entry process requires initial gp120 contact with CD4 to trigger a conformational change that promotes the exposure of gp120 domains to the chemokine coreceptor. Further binding of gp120 to the chemokine coreceptor mediates two major biological events: membrane fusion and signal transduction. HIV-1 envelope has been known to trigger a variety of signaling cascades through its binding to viral receptors (6, 7). For example, binding of gp120 can trigger the activation of Pyk2 (8), PI3K and Akt (9–11), Erk-1/2 MAPK (11), caspase-3 (12), as well as CD4/CXCR4-dependent NFAT nuclear translocation (13).

Early studies using transformed cell lines have suggested an independent role of chemokine receptor signaling in HIV infection (14–20). Nevertheless, recently, it has been demonstrated that chemokine receptor signaling plays a critical role in viral infection of primary human T cells such as the unstimulated resting CD4 T cells in the peripheral blood (21, 22). Viral binding to its chemokine coreceptor can trigger chemotactic activities leading to cofilin activation and actin rearrangement. This virus-mediated process is involved in HIV intracellular migration across the cortical actin barrier (21). The following protocols describe procedures for measuring actin cytoskeletal rearrangement and cofilin activation following the viral attachment to the chemokine coreceptor, CXCR4, on resting CD4 T cells.

2. Materials

2.1. Isolation of Human Resting CD4 T Cells from Peripheral Blood

1. RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 mg/ml) (Invitrogen, Carlsbad, CA).
2. Lymphocyte separation medium (Mediatech, Inc., Manassas, VA).
3. PBS + 0.1% BSA: 0.1 g of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) is dissolved in 100 ml of 1× PBS (no calcium, no magnesium, Invitrogen, Carlsbad, CA). The buffer is filtered through 0.45-μm filter.
4. Dynal T Cell Negative Isolation kit (Invitrogen, Carlsbad, CA).
5. Monoclonal antibodies against human CD4, CD11b, and CD19 (BD Biosciences, San Jose, CA).
6. Dynalbeads Pan Mouse IgG (Invitrogen, Carlsbad, CA).

2.2. HIV-1 Virus Preparation

1. DMEM supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA).
2. Lipofectamine 2000 (Invitrogen, Carlsbad, CA).
3. HIV indicator cell line, Rev-CEM (NIH AIDS Research & Reference Reagent Program, Cat# 11467).
4. Alliance p24 antigen ELISA Kit (Perkin Elmer, Waltham, MA).

2.3. Stimulation of Cell Receptors with HIV-1, gp120 or Magnetic Beads Conjugated with Antibodies Against CD4 and CXCR4

1. HIV-1 gp120 IIIB (Microbix Biosystems, Toronto, Canada).
2. Antibodies against human CD4 (clone PRA-T4) and CXCR4 (clone 12G5) (BD Biosciences, San Jose, CA).

2.4. Measurement of Actin Rearrangement Induced by HIV Stimulation of Chemokine Coreceptors

1. FITC-labeled phalloidin (Sigma-Aldrich, St. Louis, MO). The reagent is dissolved in DMSO at a final concentration of 0.3 mM.
2. BD Cytoperm/Cytofix buffer and BD Perm/Wash buffer (BD Bioscience, San Jose, CA).
3. 1% Paraformaldehyde in PBS (*see* [Note 1](#)).

2.5. Measurement of Cofilin Activity by Western Blot

1. SDS-NuPAGE gel, sample buffer, running buffer, transfer buffer, and nitrocellulose membrane (Invitrogen, Carlsbad, CA). Gel electrophoresis and transfer are performed as suggested by the manufacturer.
2. Tris-buffered saline (TBS): dissolve 3 g Tris base, 8 g NaCl, 2 g KCl in H₂O; adjust to pH 7.6 with HCl; adjust volume to 1,000 ml. For making TBST, add Tween 20 (Sigma-Aldrich, St. Louis, MO) to TBS to a final concentration of 0.2% (v/v).
3. Starting Block blocking buffer (Pierce, Rockford, IL).
4. Rabbit antihuman cofilin antibody and rabbit antihuman phospho-cofilin (ser3) antibody (Cell Signaling, Danvers, MA).
5. Goat antirabbit horseradish peroxidase-conjugated antibodies (KPL, Gaithersburg, MD).
6. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

3. Methods

To observe HIV-triggered signal transduction through the chemokine coreceptor, resting CD4 T cells are isolated through antibody-mediated negative depletion by removing non-CD4 and activated T cells from peripheral blood. Purified cells can be stimulated with gp120, viral particles, or antibodies to observe actin rearrangement and cofilin activation. Stimulated resting T cells need to be permeabilized and then stained with FITC-labeled phalloidin to detect actin polymerization and depolymerization. FITC-phalloidin binds only to polymeric F-actin and not to monomeric G-actin. Stained cells can be analyzed with flow cytometry to measure F-actin intensity.

Cofilin phosphorylation (inactivation) or dephosphorylation (activation) at serine 3 can be observed following stimulation of resting CD4 T cells with gp120, HIV-1, or antibodies against CXCR4. This can be accomplished through western blot using an antibody to the phosphorylated form of cofilin. For controls, on the same blot, total cofilin (phosphorylated and dephosphorylated) can also be measured with an anticofilin antibody following stripping of the antiphospho-cofilin antibody.

**3.1. Isolation of
Human Resting CD4
T Cells from
Peripheral Blood**

1. Pour peripheral blood collected from healthy donors into 50-ml Falcon tubes (up to 15 ml of blood per tube) and add an equal volume of PBS buffer to dilute the blood. Add approximately 15 ml of lymphocyte separation medium to the bottom of each tube. At room temperature, centrifuge for 20 min at $160 \times g$ with the brake off.
2. Remove approximately 20 ml of supernatant to deplete platelets, and then continue to centrifuge, this time at $350 \times g$, for an additional 20 min at room temperature with the brake off.
3. The white interface between the plasma and the lymphocyte separation medium contains the mononuclear cells. Recover and transfer the cells to a new 50-ml tube and wash them once with PBS + 0.1% BSA at 4°C by centrifugation at $400 \times g$ for 5 min.
4. Remove the supernatant and wash the cell pellet twice with cold PBS + 0.1% BSA. Count the cell number before the last centrifugation. Resuspend the cell pellet in PBS + 0.1% BSA at a concentration of 10^8 cells/ml. Transfer the cells into a 5-ml Falcon tube; add 0.2 ml of heat-inactivated FBS per ml of cells.
5. For negative depletion of non-T cells, using Dynal T cell Negative Isolation kit. Briefly, add antibody mix (20 μ l per 1×10^7 cells) and incubate at 4°C with gentle rocking for 15 min. Wash cells with PBS + 0.1% BSA, and then pellet and resuspend the cells in PBS + 0.1% BSA at a concentration of 1×10^7 cells per 900 μ l. Add prewashed depletion Dynal beads (100 μ l beads per 1×10^7 cells) and incubate for 15 min at room temperature. Add PBS + 0.1% BSA (1 ml per 1×10^7 cells) and then place on magnet for 2 min. Collect the supernatant and count the number of cells.
6. For further negative depletion of non-CD4 T cells, pellet cells and resuspend into PBS + 0.1% BSA (100 μ l per 10^7 cells), and then add 20 μ l heat-inactivated FBS per 100 μ l of cells. Add antihuman CD8 antibody (3 μ l per 10^7 cells), antihuman CD11b antibody (3 μ l per 10^7 cells), and antihuman CD19 antibody (2 μ l per 10^7 cells), and incubate at 4°C for 20 min with gentle rocking (*see* [Note 2](#)).
7. Wash cells with PBS + 0.1% BSA as described earlier to remove unbound antibodies. Resuspend cells in PBS + 0.1% BSA at a concentration of 1×10^7 cells per ml. Add prewashed Dynal beads pan antimouse IgG (four beads per cell) and incubate at 4°C for 20 min with gentle rocking. Place the tube in magnet for 2 min and then transfer cell supernatant to a new tube. Count the number of cells and pellet them by centrifugation as described earlier.

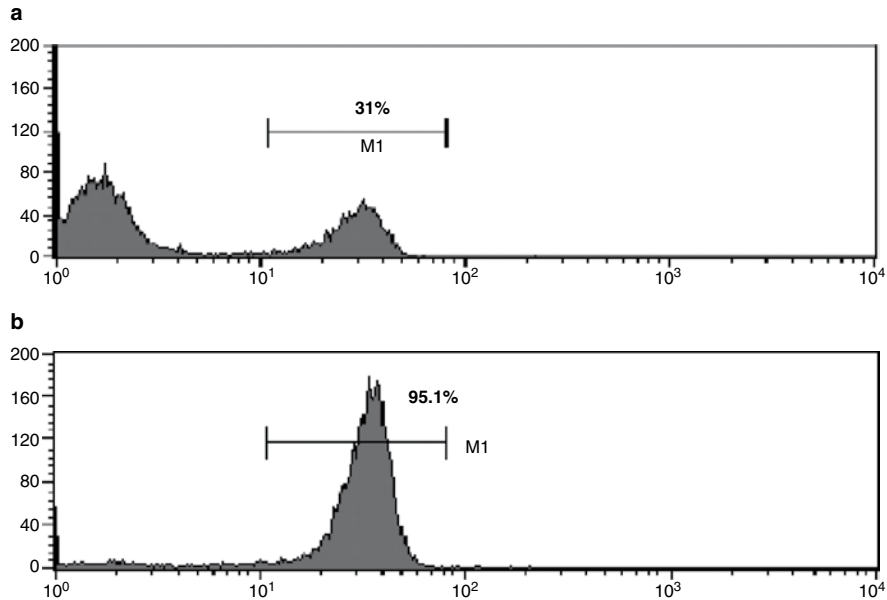


Fig. 1. Analysis of resting CD4 T cells purified from peripheral blood. Cells were purified from peripheral blood through antibody-mediated negative depletion. Shown are cells stained with FITC-labeled antihuman CD4 antibody before (a) and after (b) purification. In this example, unpurified blood mononuclear cells contain 31% of CD4-positive cells, and following purification, the CD4-positive cells were enriched to 95.1%.

8. Resuspend cells in RPMI 1640 + 10% FBS at a concentration of 1×10^6 cell per ml. Culture cells in flasks at 37°C , 5% CO_2 overnight before treatment. An analysis of the purity of CD4 T cells is shown in **Fig. 1**.

3.2. HIV-1 Virus Preparation

1. Prepare virus stocks of HIV-1 by transfection of HeLa or HEK293T cells with cloned proviral DNA (*see Note 3*).
2. Culture cells in DMEM + 10% FBS at 37°C , 5% CO_2 until they are 80–90% confluent. One day before transfection, dislodge cells from T75 flasks and plate them into 10-cm petridishes at 3×10^6 cells/dish.
3. Grow cells in petridishes at 37°C for 12–24 h until they are 80% confluent. Remove medium from each petridish, rinse with serum-free DMEM, and then add 5 ml serum-free DMEM.
4. For transfection of cells in each petridish, mix 20 μg of plasmid DNA (such as pNL4-3) with 1.5 ml serum-free DMEM in a tube.
5. In another tube, mix 60 μl of Lipofectamine 2000 with 1.5 ml serum-free DMEM, and then incubate at room temperature for 5 min.

6. Mix DNA with Lipofectamine 2000. The total volume is 3 ml. Incubate at room temperature for 20 min.
7. Add 3 ml of the DNA/Lipofectamine 2000 mixture to each petridish and incubate for 5–6 h. Remove the transfection supernatant and add DMEM + 10% FSC (10 ml/dish) to continuously culture the transfected cells.
8. Harvest the supernatant at 48 h post transfection by pelleting cells at $400 \times g$ for 10 min. Save the supernatant and filter it through a 0.45- μ M filter. Store the supernatant containing HIV-1 at -80°C in 0.5–1.0 ml aliquots.
9. Virus titer (TCID_{50}) can be measured by infection of a Rev-dependent indicator cell line, Rev-CEM (23) (see Note 4). An example of the result using Rev-CEM is shown in Fig. 2.
10. Levels of viral p24 in the supernatant can also be measured using the Perkin Elmer Alliance p24 Antigen ELISA Kit.

3.3. Stimulation of Cell Receptors with HIV-1, gp120, or Magnetic Beads Conjugated with Antibodies Against CD4 and CXCR4

1. For the stimulation of resting CD4 T cells with HIV-1, normally $10^{3.5}$ – $10^{4.5}$ TCID_{50} units of HIV-1 are used to treat 10^6 cells (2 – 4×10^6 per ml in medium) in 2-ml round bottom tubes. Cells are incubated at 37°C in an Eppendorf Thermomixer with gentle agitation (600–1,200 rpm) to prevent cells from settling at the bottom. The use of low viral dosages is important for the observation of $\text{G}\alpha\text{i}$ signaling. It has been demonstrated that at high chemokine concentrations, chemokine receptors can be promiscuously coupled to both PTX-sensitive $\text{G}\alpha\text{i}$ and insensitive $\text{G}\alpha\text{q}$ (24, 25). The treatment duration can last from 10 s to 2 h.

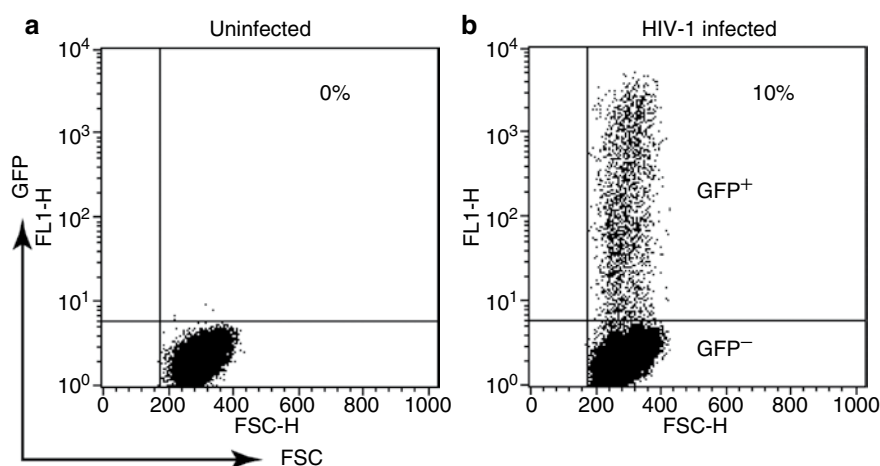


Fig. 2. Measurement of HIV-1 titer using Rev-CEM. Rev-CEM cells were infected with HIV-1 for 48 h, and then analyzed with flow cytometry to measure the number of GFP-positive cells. In this example, the HIV-1 preparation can productively infect 10% Rev-CEM cells (b). Virus titer can be calculated based on the percentage of GFP-positive cells. Uninfected Rev-CEM cells did not generate any GFP-positive cells (a).

2. For the stimulation of resting CD4 T cells with purified gp120 protein, usually 5 pM to 50 nM gp120 are used to treat 10^6 cells at a concentration of $2\text{--}4 \times 10^6$ cells/ml. As described earlier, the treatment duration can last from 10 s to 2 h.
3. Resting CD4 T cells can also be stimulated with antibodies against the CD4 or CXCR4 receptors (*see Note 5*). For conjugation of antibodies to magnetic beads, 10 μ g of antibodies is conjugated with 4×10^8 Dynal beads for 30 min at room temperature. Free antibodies are washed away with PBS + 0.5% BSA, and the beads are resuspended in 1 ml of PBS + 0.5% BSA. For stimulation of resting CD4 T cells, antibody-conjugated beads are washed twice, and then added to cell culture at a density of two to four beads per cell.

**3.4. Measurement
of Actin Rearrange-
ment Induced by HIV
Stimulation
of Chemokine
Coreceptors**

1. One to two million cells are resuspended in medium at a density between 2 and 4×10^6 cells/ml. Cells are stimulated with HIV-1, gp120, or antibody conjugated magnetic beads as described earlier.
2. F-actin staining using FITC-labeled phalloidin is carried out using $1\text{--}2 \times 10^6$ cells (*see Note 6*). Stimulated cells are pelleted in a microcentrifuge at $300 \times g$ for 2 min. The supernatant is removed and cells are fixed and permeabilized with 400 μ l of BD Cytoperm/Cytofix buffer for 20 min at room temperature (*see Note 7*). Cells are washed with 2 ml cold BD Perm/Wash buffer twice, pelleted at $500 \times g$ for 5 min at 4°C, followed by staining in the residual BD Perm/Wash buffer (approximately 100–150 μ l) with 5 μ l of 0.3 mM FITC-labeled phalloidin for 30 min on ice in the dark.
3. After washing twice with 2 ml cold BD Perm/Wash buffer, cells are pelleted as earlier, resuspended in 1% paraformaldehyde, and analyzed on a flow cytometer. An example of the F-actin staining following treatment is shown in Fig. 3.

**3.5. Measurement
of Cofilin Activity by
Western Blot**

1. Following stimulation, one to two million CD4 T cells are pelleted at $300 \times g$ for 2 min in a microcentrifuge. Cells are lysed in NuPAGE LDS sample buffer. The cell lysates can be stored at -80°C .
2. Before SDS-PAGE gel electrophoresis, cell lysates are sonicated to break DNA and reduce viscosity. Samples are heated at 70°C for 10 min, centrifuged at the maximal speed in a microcentrifuge for 5 min, and then loaded and separated by SDS-PAGE.
3. Following gel electrophoresis, proteins are transferred onto nitrocellulose membranes. The membranes are washed in TBST for 5 min and then blocked for 30 min at room temperature with Starting Block blocking buffer. The blots are incubated

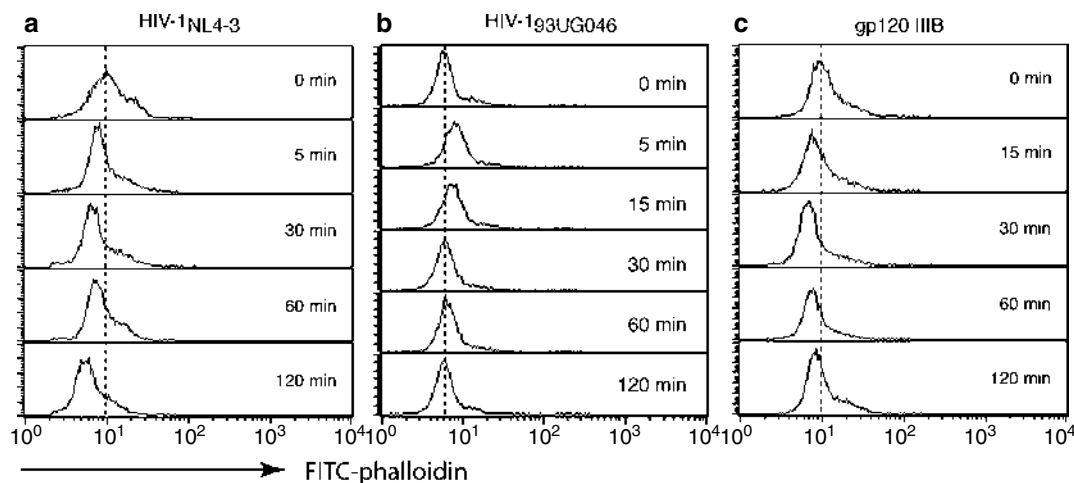


Fig. 3. HIV-1 envelope signaling triggers actin rearrangement. Resting CD4 T cells were treated with a laboratory-adapted viral strain, HIV-1_{NL4-3} (a), or with a primary viral isolate, HIV-1_{93UG046} (b), or with gp120 IIIB (100 pM) (c) for various times, fixed and permeabilized, and then stained with FITC-phalloidin for F-actin and analyzed by flow cytometry. Shown are histograms of F-actin staining.

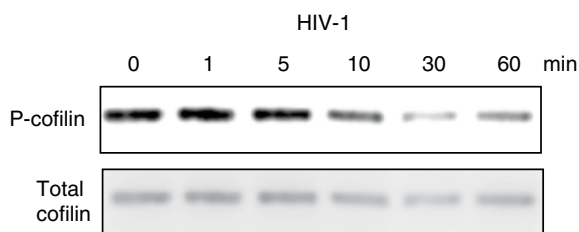


Fig. 4. Cofilin activation in HIV-1 infection of resting CD4 T cells. Resting CD4 T cells were purified and cultured overnight without stimulation. Cells were treated with HIV-1 for various times as described in the protocol. One-tenth of the cell lysates were analyzed by SDS-PAGE and western blot using antibodies against human phospho-cofilin (*top panel*) or total cofilin (*bottom panel*).

with either a rabbit anticyofilin antibody (1:1,000 dilution) or a rabbit antiphospho-cofilin (ser3) antibody (1:1,000 dilution) diluted in TBST + 5% BSA and rocked at room temperature for 1 h or overnight at 4°C.

4. The blots are washed three times for 15 min each and then incubated with goat antirabbit horseradish peroxidase-conjugated antibodies diluted in TBST + 2.5% skim milk (1:5,000) for 1 h.
5. The blots are washed with TBST again three times for 15 min each and then developed with SuperSignal West Femto Maximum Sensitivity Substrate. Images are captured with a CCD camera such as the FluorChem 9900 Imaging System from Alpha Innotech.
6. The blot can be stripped and reprobed with antibodies against total cofilin as a control (*see Note 8*). An example of the western blot result is shown in **Fig. 4**.

4. Notes

1. For preparing 1% paraformaldehyde in PBS, all reagents and the pH meter should be placed in a chemical hood. Inside the hood, dissolve 1 g of paraformaldehyde in approximately 90 ml of distilled water by adjusting pH to 10 with 1 N NaOH, and then adjust pH to 7 with HCl. Add 10 ml 10 × PBS and use distilled water to make the final volume to 100 ml.
2. For purification of resting CD4 T cells by negative depletion, two rounds of deletion will generate higher purity than a single round of depletion.
3. For transfection of cells, both HeLa and HEK293T cells can be used. However, between these two commonly used cell lines, HeLa cells generate lower virus titers and less viral-free gp120 than HEK293T cells. For observing signaling at low viral dosages, we usually use HeLa cells to produce viruses. For observing strong actin polymerization in high viral dosages, HEK293T cells can be used and the virus can be harvested at day 3.
4. The Rev-CEM cell line has no background GFP expression and gives viral titers close to those obtained using PBMC (23). For measuring viral titer, Rev-CEM can be used for TCID₅₀ assay or for flow cytometry. For TCID₅₀ assay, cells are resuspended into 1×10^6 cells/ml, and 100 μ l of cells is used in each well of 24-well culture plates. Virus supernatant is serially diluted and 50 μ l of the virus is added into each row (six wells per row). The infection is carried out for 2 h, and then 1 ml fresh medium is added. The infected cells are cultured for 5–7 days and the number of GFP-positive wells is counted and calculated using the Reed–Muench method (23). For using flow cytometry to measure viral titer, approximately $1\text{--}5 \times 10^5$ cells can be infected with 100 μ l of virus, and following infection for 2 h, cells were washed once and resuspended into 1 ml fresh medium. To prevent secondary infection, protease inhibitors can be added and infected cells are cultured for 2–3 days and analyzed by flow cytometry to measure the percentage of infected cells.
5. Monoclonal antibodies against human CD4 (clone PRA-T4) or CXCR4 (clone 12G5) can be used to stimulate resting T cells. These antibodies are selected for their shared epitopes with gp120. The CD4 antibody, clone RPA-T4, binds to the D1 domain of the CD4 antigen and is capable of blocking gp120 binding to CD4 (2), whereas the anti-CXCR4 antibody, clone 12G5, interacts with the CXCR4 extracellular loops 1 and 2, which partially overlap domains for HIV-1 coreceptor function (26). The 12G5 antibody has also been shown to block HIV-1-mediated cell fusion (27) and CD4-independent HIV-2 infection (28).

6. It is important to use a sufficient number of cells (at least one million) to obtain reliable and reproducible staining. Multiple washing and pelleting steps tend to decrease the number of cells and to deform them. A significant number of cells are not appropriate for flow cytometry analyses. These deformed cells and cell debris can be gated out based on the forward scatter and sideward scatter during the analysis.
7. After the first centrifugation at $300 \times g$ for 2 min in a microcentrifuge, remove the supernatant with a P1000 pipette tip. Do it gently and do not touch the cell pellet. Leave some liquid at the bottom and lightly tapping the tube to resuspend cells, then add approximately 400 μ l Cytoperm/Cytofix buffer.
8. For stripping, it is usually difficult to completely strip the antiphospho-cofilin antibody, to avoid interference from previous staining, you may need to prepare two identical protein gels and stain them for phospho-cofilin and total cofilin separately. Alternatively, to serve as a loading control for total proteins, the blot can be reprobed with a different antibody such as the rabbit antibodies against human GAPDH (Abcam, Cambridge, MA).

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Chapter 22

Spatiotemporal Stimulation of Single Cells Using Flow Photolysis

Carsten Beta

Summary

Quantitative studies of chemotactic signaling require experimental techniques that can expose single cells to chemical stimuli with high resolution in both space and time. Recently, we have introduced the method of flow photolysis (Anal. Chem. 79:3940–3944, 2007), which combines microfluidic techniques with the photochemical release of caged compounds. This method allows us to tailor chemical stimuli on the length scale of individual cells with subsecond temporal resolution. In this chapter, we provide a detailed protocol for the setup of flow photolysis experiments and exemplify this versatile approach by initiating membrane translocation of fluorescent fusion proteins in chemotactic *Dictyostelium discoideum* cells.

Key words: Caged compounds, Photoactivation, Microfluidics, Soft lithography, Laser scanning confocal microscopy, Chemotaxis, Directional sensing, *Dictyostelium discoideum*

1. Introduction

The chemotactic movement of cells under the influence of directional chemical cues is a key factor in many biomedical processes ranging from wound healing to cancer metastasis and morphogenesis (2). While the molecular basis of bacterial chemotaxis is well understood, less is known about the more complex pathways that control chemotactic signaling in eukaryotic cells (3). Since the late 1990s, the use of fluorescent fusion proteins has stimulated much progress in identifying the molecular players that are involved in the early stages of chemotactic sensing (4). However, a generally accepted picture of directional sensing in eukaryotic cells is still missing. Different models have been proposed that describe the initial symmetry breaking observed in the internal

protein distribution of a cell exposed to an extracellular chemoattractant gradient, *see e.g. refs. 5–8*. While all these models account for an asymmetric distribution of various signaling components under gradient conditions, they differ in their quantitative predictions of how the dynamics of such components depend on the gradient steepness, midpoint concentration, and gradient exposure time. In order to verify or falsify different competing models of directional sensing, experimental techniques are required that enable us to expose individual chemotactic cells to well-defined gradient stimuli with high spatiotemporal resolution.

Over the last decade, microfluidic techniques have been established as a versatile platform to perform life cell experiments under well-controlled conditions (9). To investigate directional responses, stable concentration profiles of chemotactic factors can be generated in microfluidic gradient mixers, *see e.g. refs. 10, 11*. Compared to classical, diffusion-based gradient chambers, microfluidic mixers provide an increased degree of spatial control. However, transient times for built-up and switching of gradient profiles are on the order of minutes. On the other hand, the first protein translocation events that are involved in chemotactic sensing can be observed only a few seconds after exposure to the external stimulus. The lack of sufficient temporal resolution in conventional microdevices has hampered a quantitative investigation of many of these fast intracellular signaling events.

Attempts have been made to increase the temporal resolution, and switching times of around 5 s were achieved in devices based on membrane valves (12). Recently, we have introduced the flow photolysis approach, an experimental method to stimulate single cells with subsecond temporal resolution (1). In particular, transient times for the built-up of gradient profiles could be reduced to below 1 s. Flow photolysis is based on the light-induced release of caged signaling substances in a microfluidic flow chamber. The underlying idea is displayed in Fig. 1a. Cells are placed in a microfluidic channel under a constant, gentle flow

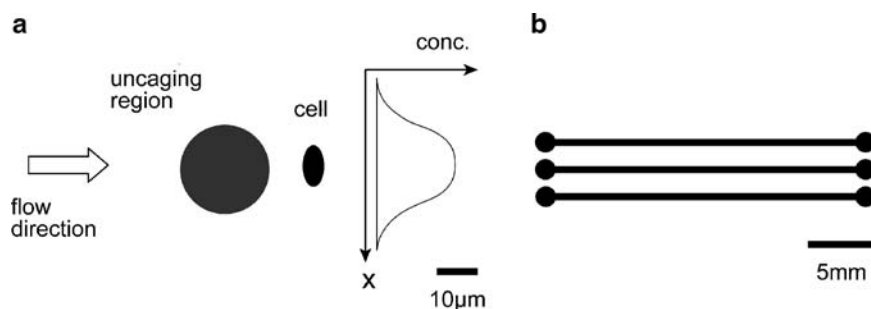


Fig. 1. The flow photolysis setup. (a) Principle of flow photolysis. The caged signaling compound is transported by a gentle flow of buffer from *left to right*. The signaling substance is photochemically released in a confined photo-uncaging region and transported by the fluid flow across the cell downstream. (b) Example layout of a microfluidic channel device, *top view*. The *shaded* structures are 25 μm high.

of buffer that carries the biologically inert, caged form of a signaling substance. Cell stimulation can be initiated by illuminating a confined region in the fluid flow upstream of the cell. Inside this region, the caged compound is released and transported across the cell by the fluid flow. Due to the short distance between the uncaging region and the position of the cell, rapid switching times are achieved. Moreover, through the interplay of fluid flow and light source geometry, a wide variety of concentration profiles can be generated. Note that this method is limited to signaling substances that are available in a photoactivatable caged form. However, much progress has been made in recent years in the preparation and handling of caged compounds. A large variety of photoactivatable chemicals is available commercially, and detailed protocols are at hand for the synthesis of less commonly used caged substances (13).

In the following, we present a detailed description of how to set up and operate a flow photolysis experiment. Our protocol consists of three parts. First, a microfluidic chamber is fabricated using soft lithography (14, 15). Second, the microdevice is mounted on a confocal laser scanning microscope, loaded with cells, and connected to a precision pumping system. Third, cells of the social amoeba *Dictyostelium discoideum*, one of the most prominent model organisms to study eukaryotic cell motility and chemotaxis (16, 17), are employed to exemplify our method. We initiate intracellular protein translocation in chemotactic *Dictyostelium* cells (plaval) via photocleavage of DMNB-caged cyclic adenosine 3',5'-monophosphate (cAMP) (18).

2. Materials

2.1. Soft Lithography

1. Master wafer (4 in. in diameter) carrying a microstructure of straight, parallel channels (see Note 1 and 2). The channel structure is 25 μm high, 500 μm wide, and about 2 cm long (see Fig. 1b).
2. Acetone, isopropanol, compressed air or nitrogen.
3. Balance, weigh boats, ScotchTM tape, sharp knife.
4. Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning).
5. Desiccator, vacuum pump, oven.
6. Dispensing needles, 19 GA $\times$ 1 in. (McMaster) (see Note 3).
7. Glass coverslips, 24 $\times$ 60 mm, No. 1 (Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG).
8. Plasma cleaner, PDC-002 (Harrick Plasma).

2.2. Device Setup

1. Teflon tubing, 39241 (Novodirect GmbH).
2. 500- μ L gas-tight glass syringes, 1750 TTLX (Hamilton Bonaduz AG).
3. Dispensing needles, 19 GA $\times$ 1/2 in. (McMaster).
4. Syringe pump PHD 2000 (Harvard Apparatus Inc.).
5. Phosphate buffer: 2 g/L KH_2PO_4 , 0.36 g/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 6.0.
6. 4,5-dimethoxy-2-nitrobenzyl (DMNB)-caged cyclic adenosine 3',5'-monophosphate (cAMP), dextran conjugate, 3,000 MW (Invitrogen Corp.).

2.3. Single-Cell Stimulation: Example

1. Cell culture equipment (*see* [Note 4](#)).
2. HL5 medium: 7 g/L yeast extract, 14 g/L peptone, 0.5 g/L KH_2PO_4 , 0.5 g/L Na_2HPO_4 .
3. Inverted confocal laser scanning microscope, Fluoview FV1000 (Olympus Corp.). The microscope is equipped with an additional, separate scanning unit to move a 405-nm laser (25 mW, FV5-LD405, Olympus Corp., *see* [Note 5](#)) independent of the imaging lasers inside the field of view (*see* [Note 6](#)).

3. Methods
3.1. Soft Lithography

Flow photolysis experiments are carried out in microfluidic channels (*see* [Note 7](#)). We use soft lithography ([14](#), [19](#)), a well-established polymer molding procedure, to build our microdevices. The following steps are carried out in the clean and dry environment of a laminar flow bench.

1. Rinse the master wafer with acetone, isopropanol, and purified water. Dry the wafer under a stream of clean compressed air or nitrogen.
2. On a balance, pour approximately 70 g PDMS into a clean weigh boat. Add curing agent in a ratio of 1:10, i.e., about 7 g. Thoroughly mix elastomer and curing agent by stirring (*see* [Note 8](#)).
3. Place the clean and dry master wafer in another weigh boat and pour the mixture of PDMS and curing agent on top.
4. Place the weigh boat with master and PDMS in a desiccator and evacuate for about 45 min to remove all gas bubbles (*see* [Note 9](#)). Spilling of PDMS due to a large number of gas bubbles can be prevented by shortly releasing the vacuum from time to time.

5. Place the weigh boat with master and degassed PDMS in an oven and bake for 1 h at about 65°C. Cool back to room temperature.
6. Cut the PDMS around an individual microstructure with a sharp knife. Peel the PDMS off the microstructure carefully and protect the molded side of the PDMS with Scotch™ tape.
7. Use a 19-gauge, clean stainless steel needle with a polished tip to punch holes into the PDMS for the inlets and outlets. Always withdraw the needle from the PDMS slowly and carefully as deformation and large stresses may induce cracks in the PDMS.
8. Place the molded piece of PDMS and a new, clean, and dry cover slip in the glass cavity of the plasma cleaner (*see Note 10*). Do not touch the surfaces that will be assembled later, not even with gloves. Load the PDMS with the molded surface pointing upward and remove the tape inside the plasma cleaner cavity. Close the plasma cleaner.
9. Evacuate the plasma cleaner. After about 1 min, turn on the radio frequency. A blue/violet plasma should be visible inside the cavity of the plasma cleaner (if not, evacuate longer).
10. Carefully let some air leak into the plasma cleaner via the needle valve in the door until the color of the plasma has changed to deep pink. Operate under these conditions for about 3 min, then turn off.
11. Open the door, pick up the glass slide with a tweezer, and turn it onto the molded side of the PDMS, so that the surfaces of the glass and the PDMS that were exposed to the plasma come into contact. They should bond immediately and no air should get trapped in between them. If bonding does not occur spontaneously, you may press gently.

3.2. Device Setup

After plasma cleaning and device assembly, transfer the device to the microscope and use as soon as possible (*see Note 11*).

1. Prepare a 1-mM stock solution of DMNB-caged cAMP in dimethylsulfoxide (DMSO). Keep frozen at -20°C and protect from light. Prepare a 10-μM dilution in phosphate buffer for the experiment.
2. Place a drop of phosphate buffer on one of the inlets of the PDMS microdevice. The liquid should start flowing into the channel, immediately filling the entire device (*see Note 12*).
3. Fill two gas-tight glass syringes, one with a solution of 10 μM DMNB-caged cAMP in phosphate buffer, the other one with cell suspension (*see Note 13*). Connect the Teflon tubing to the syringes via dispensing needles and fill the tubing until a drop of liquid is appearing at the open end. Mount the

syringe with caged cAMP on the Hamilton syringe pump. Protect the syringe from light by covering with aluminum foil.

4. Connect the cell suspension syringe to the inlet where the initial drop of phosphate buffer was placed by inserting the tubing into the prepunched hole in the PDMS.
5. Manually inject the cell suspension into the microfluidic channel until a drop of suspension is forming at the other inlet.
6. Remove the cell-loading syringe and connect the syringe with caged cAMP at the opposite inlet by merging the drop of liquid at the inlet with the drop at the open end of the syringe tubing.
7. Give the cells about 15 min to settle and attach (*see Note 14*). Set the pump to a pumping rate of 5 $\mu\text{L}/\text{h}$, and start the flow of caged cAMP. This corresponds to a flow speed of about 110 $\mu\text{m}/\text{s}$ for the given channel dimensions (*see Note 15*). After about 30 min, single-cell stimulation experiments can start.

3.3. Single-Cell Stimulation: Example

We demonstrate the flow photolysis approach in single-cell experiments on the social amoeba *D. discoideum*. Following light-induced chemoattractant release, membrane translocation of a fluorescently labeled signaling protein is initiated.

1. *D. discoideum* cells of the AX3-derived strain WF38 are used that express a green fluorescent protein (GFP) fused to the pleckstrin homology (PH) domain of the cytosolic regulator of adenylyl cyclase (CRAC). Cells are grown in Petri dishes under HL5 nutrient solution.
2. To establish chemotactic behavior, cells are washed with phosphate buffer and starved for 7–8 h in a Petri dish under a thin phosphate buffer film. Prior to the experiment, cells are gently resuspended from the bottom of the Petri dish with a pipette and loaded into the microfluidic chamber as described in **Subheading 3.2**.
3. The single-cell measurements are started at the far end of the channel close to the outlet. Work your way up toward the inlet through which the caged cAMP solution is infused. In this way, it can be ensured that a chosen cell has not been hit by the cAMP release of a previous stimulation experiment.
4. Choose the cell on which a stimulation experiment shall be performed. Place the cell in the center of the field of view by movement of the microscope stage. Avoid cells that are located in a crowded environment as the flow field in their vicinity may be strongly disturbed.

- Choose position and shape of the uncaging region with respect to the cell (*see* [Note 16](#)). This will determine the concentration profile the cell is exposed to if photo-uncaging is initiated (*see* [Note 17](#)). An example of a uniform stimulus can be seen in [Fig. 2](#). Here, a rectangular region upstream of the cell is scanned by the photo-uncaging laser. A simple laser point source or a triangular-shaped uncaging region can be employed for generation of concentration gradients as illustrated in [Fig. 3a](#) and [b](#), respectively.

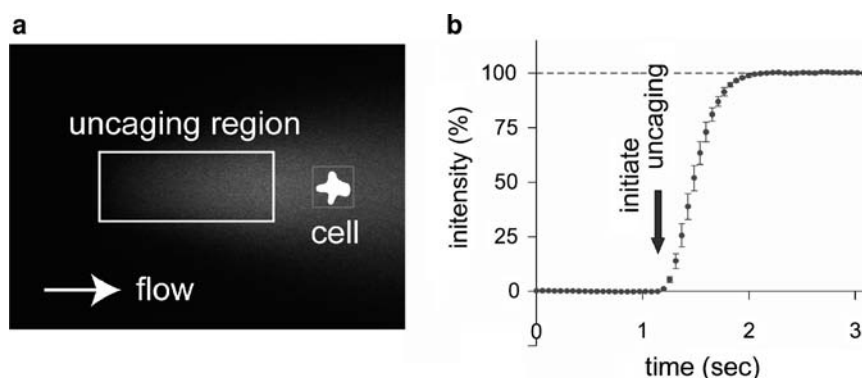


Fig. 2. Uniform stimulus and switching time. (a) A solution of 10 μM DMNB-caged fluorescein is transported by the flow from *left to right*. Photochemical release of the dye is initiated inside the *white rectangle* by rapid scanning of a 405-nm laser. (b) The fluorescence intensity is measured in a *small rectangular region* around the position of the cell in (a). The average intensity is shown in the course of time. Less than 1 s is required for switching of the dye concentration at the location of the cell. Reproduced with permission from [ref. 1](#). Copyright 2007 American Chemical Society.

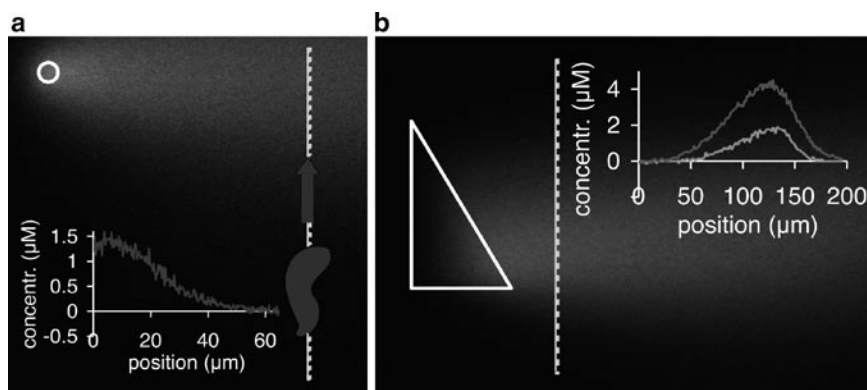


Fig. 3. Gradient stimuli. (a) Gradient generation with a laser point source. A 10- μM solution of DMNB-caged fluorescein is released in the *upper left corner* of the field of view. The uncaged substance spreads downstream due to diffusion. The *inset* shows the dye concentration along the *dashed line*. (b) Gradient generation by a *triangular* uncaging region. DMNB-caged fluorescein is released inside a *triangular* uncaging region by rapid scanning of a 405-nm laser. The resulting concentration profile along the *dashed line* is shown in the *inset* for two different flow speeds (*dark gray*: 67 $\mu\text{m/s}$; *light gray*: 133 $\mu\text{m/s}$). In contrast to the laser point source, a well-defined shape of the concentration profile can be imposed by tuning the geometry of the uncaging region. Reproduced with permission from [ref. 1](#). Copyright 2007 American Chemical Society.

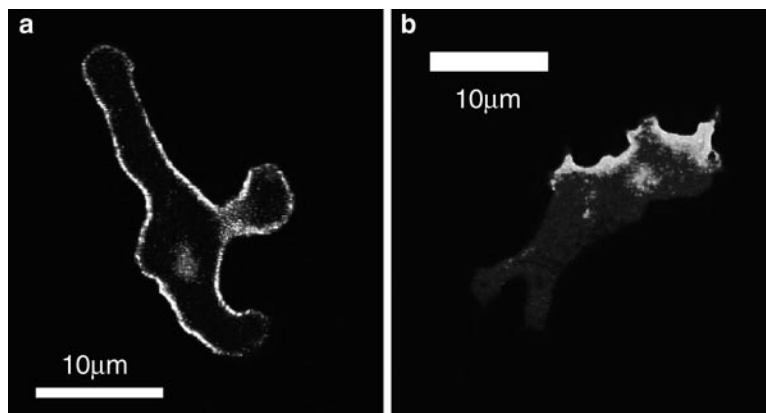


Fig. 4. Translocation of CRAC-GFP in chemotactic *Dictyostelium* cells. (a) Response to a uniform stimulus of cAMP, generated as illustrated in Fig. 2a. Translocation occurs around the entire perimeter of the cell. (b) Directional response to a gradient of cAMP, generated as illustrated in Fig. 3a. The gradient direction is from *bottom* (low concentration) to *top* (high concentration). Translocation occurs preferentially at the side of the cell toward higher cAMP concentrations. Reproduced with permission from ref. 1. Copyright 2007 American Chemical Society.

6. Start time-lapse confocal imaging. In the case of PH-domain translocation in *D. discoideum*, it is sufficient to record one image per second. At the desired point in time during image acquisition, photo-uncaging is initiated by rapidly scanning the 405 nm photo-uncaging laser across the uncaging region. Examples of PH-domain translocation events under uniform or directional translocation can be seen in Fig. 4a and b, respectively.
7. The time-lapse movies of single-cell stimulation events are subsequently computer processed to quantify the response behavior.

4. Notes

1. The desired layout of the microstructure is designed using a computer-assisted design (CAD) software. Based on the resulting CAD file, the master wafer can be generated in a clean room environment by photolithographic patterning of a photoresist-coated Si-wafer. For standard photolithography protocols see e.g., refs. 14, 20. Alternatively, the structured master wafer can be bought from a microfabrication service facility that custom fabricates the master based on the individually provided CAD file.

2. It is advantageous to choose a layout that yields more than one microchannel per device. An example with three parallel channels is displayed in **Fig. 1b**. This ensures that an intact channel is available for the experiment, even if one (or two) of the channels are damaged by production errors.
3. Sharpening of the needle tip by manual polishing facilitates punching of the inlets and helps to prevent the emergence of cracks in the PDMS. Cracks close to the inlets can become a major problem. They may cause leakage of an inlet connection and render a device useless.
4. The required equipment depends on the type of cells that will be investigated in the flow photolysis stimulation experiments. In the case of *Dictyostelium*, only the most basic cell culture instrumentation – flow bench, centrifuge, autoclave, freezer, pipettes, and plastic consumables – is required.
5. For most cages, wavelengths between 330 and 380 nm are optimal to initiate photocleavage. Here, we decided to use a 405-nm laser. Although uncaging is less efficient in this case, it has the advantage that this laser is typically available on life cell imaging systems to perform photobleaching experiments. UV lasers, on the other hand, are much more expensive and are not part of standard imaging setups.
6. The flow photolysis method presented here was developed on an Olympus Fluoview FV1000 microscope. However, other laser scanning confocal microscopes may work equally well if similarly equipped with a separate scanning unit.
7. In principle, flow photolysis is not limited to microfluidic devices. Also larger perfusion chambers could be used. However, microdevices have the advantage that only small amounts of the expensive caged compounds are required to carry out the stimulation experiments. Moreover, excellent control of flow properties is easily obtained on the length scale of individual cells.
8. The required amount of PDMS depends on the diameter of the master wafer and on the size of the weigh boat. Choose the amount such that the layer of PDMS on the wafer is about 1 cm in thickness. Do not make it too thick because this may complicate the punching of inlets and increases the risk of trapping air bubbles when filling the device later. Do not make it too thin either to ensure that there is enough room for inserting the tubing.
9. Take care that no gas bubbles are left underneath the wafer; otherwise, the wafer might break when cutting the PDMS.
10. Some protocols recommend washing the glass slides prior to device assembly. However, in our case, new glass slides taken directly from stock worked fine.

11. The PDMS devices can be kept in purified water for later use. However, storage increases the chances of air bubbles and dirt getting trapped inside the channel.
12. It is advantageous to fill the channels as soon as possible after device assembly. The PDMS stays hydrophilic for about half an hour. During this time, the channels fill easily due to the combined action of hydrophilicity and capillarity, so that bubble formation is reduced. Air bubbles can become a major problem as they cause unstable flow profiles. Make sure that no air bubbles are trapped inside when filling the syringes and connecting the tubing.
13. For calibration, an identical photo-uncaging experiment is performed with a 10- μ M solution of 4,5-dimethoxy-2-nitrobenzyl (DMNB)-caged fluorescein (dextran conjugate, 3,000 MW, Invitrogen Corp.). The amount of released dye is determined by comparison with the fluorescence from a solution in which all dye has been uncaged. Since we chose a dye that carries the same caging group as the caged cAMP, we can expect similar uncaging kinetics. An elegant variant of this calibration protocol is the use of caged compounds that carry a fluorescent cage, e.g., the [6,7-bis(carboxymethoxy) coumarin-4-yl]methyl (BCMCM) ester of cAMP (21).
14. The present protocol is designed for experiments with *Dicystostelium* cells. For other cell types different adhesion times might be required. In the case of nonadhering or weakly adhering cells, the protocol needs to be extended to take the use of surface coatings into account that immobilize the cells on the walls of the microfluidic channel.
15. In flow photolysis experiments, and also in other flow chambers for gradient generation, the flow speed has to be carefully controlled and should remain within a certain optimal range to avoid flow-induced deviations from the desired gradient profile. In particular, if flow speeds are strongly increased, the gradient exposure on the cell surface will be reduced as compared to the imposed profile in the undisturbed flow. For a detailed analysis of flow effects in chemotaxis assays, see ref. 22.
16. The details of how the photo-uncaging region is defined depend on the specific software that controls the microscope.
17. In general, the spatial distribution of the uncaged chemical is determined by (1) the concentration of the caged compound in the flow, (2) its uncaging kinetics, (3) the diffusion constant of the uncaged compound, (4) the size and shape of the illuminated region, (5) the intensity of the uncaging light source, and (6) the flow speed; see also ref. 1.

Acknowledgments

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Chapter 23

Spatiotemporal Regulation of Ras-GTPases During Chemotaxis

Atsuo T. Sasaki and Richard A. Firtel

Summary

Many eukaryotic cells can elicit intracellular signaling relays to produce pseudopodia and move up to the chemoattractant gradient (chemotaxis) or move randomly in the absence of extracellular stimuli and nutrients (random movement). A precise spatiotemporal regulation of Ras-GTPases, such as Ras and Rap, is crucial to induce pseudopodia formation and cellular adhesion during the chemotaxis and random movement. Here, we describe biochemical and real-time imaging methods for using *Dictyostelium* to understand the signaling events important for chemotaxis and random cell movement. The chapter includes (1) a biochemical method to assess Ras and Rap1 activation in response to chemoattractant, (2) an imaging method to detect endogenous Ras and Rap1 activation in moving cells, and (3) a simultaneous imaging method to decipher the precise order and localization of these signaling events. With a combination of powerful *Dictyostelium* genetics, these methods will facilitate to elucidate a dynamic activation of Ras proteins and their inter relay with other signaling molecules during chemotaxis and random movement.

Key words: G-protein, Ras, Rap; PI3K, Chemotaxis, Directional movement, Cell polarity, Cell adhesion, Random movement, Cytokinesis, Live imaging, *Dictyostelium*

1. Introduction

Chemotaxis is a remarkable process in which cells sense an external chemical gradient and transmit it to the inside of the cell. Internal signaling molecules amplify this signal and trigger effector proteins that lead to the remodeling of the actin cytoskeleton and cell adhesion, and to cell migration (1–3). In amoeboid chemotaxis, which includes that of *Dictyostelium* cells and leukocytes, chemoattractants bind to and activate GPCRs (G-protein-coupled receptors) that signal downstream effectors through

heterotrimeric G-proteins (4–9). In addition to chemotaxis, many eukaryotic cells have an intrinsic ability to produce pseudopodia and can move in the absence of chemoattractants or nutrients (random cell movement) (10–11). The random cell migration enables cells to explore their environment and is linked to metastasis of tumor cells (12–14).

Ras family proteins are monomeric GTPases that act as biological switches by cycling between GDP-bound inactive and GTP-bound active states (5, 15, 16). Most of the Ras proteins are in the inactive GDP-bound form in resting cells; however, guanine nucleotide exchange factors (GEFs) catalyze GTP loading on Ras in response to upstream stimulation (17). GTPase-activating proteins (RasGAPs) negatively regulate Ras activation by accelerating GTP hydrolysis. GTP-bound Ras induces downstream signaling by direct interaction with the effector molecules. Various Ras effectors have been identified, and among which Raf kinases, PI3K, and RalGDS are important mediators of the tumorigenic function of Ras (18). Structural analyses demonstrate that GTP loading drastically changes the conformation of the Switch I and Switch II regions of Ras, and these regions are involved in high-affinity binding to the effector molecules (19, 20). Two decades of intensive studies provided compelling evidence for Ras having a critical role in cell growth, differentiation, and survival. However, the importance of Ras in chemotaxis, cell polarization, and directional sensing remained unclear until very recently, due to a lack of proper tools and assay systems. The *in vivo* time-lapse imaging analyses described in this chapter allowed us to determine that Ras is rapidly and transiently activated in response to chemoattractant. In chemotaxing cells, activated Ras is restricted to activate effectors, including PI3K, at the leading edge whereas total Ras protein is uniformly distributed along the plasma membrane. We discovered a novel dynamic feedback activation of Ras and PI3K which is essential for the proper chemotaxis and random movement (Fig. 1)(10, 21).

Rap1 is one of the closest Ras subfamily proteins. Accumulating evidence implicates Rap1 as a critical regulator for cell adhesion, cell–cell junction formation, cell polarity, vesicle trafficking, and phagocytosis (22, 23). Like Ras, Rap1 is activated by diverse extracellular stimuli through distinctive Rap-specific GEFs and turned off by Rap-specific GAPs (RapGAPs). Although Rap1 shares the identical effector domain sequence with Ras, Rap1 and Ras differ in the affinity to interact with their effector molecules. For example, Ras binds to the Ras-binding domain of human Raf1 kinase (RBD-Raf1) with much higher affinity than Rap1 does. On the other hand, active-Rap1 binds to RBD-RalGDS much stronger than Ras does (24, 25). These two RBDs clearly distinguish between Ras and Rap1 in *Dictyostelium*, which we have demonstrated by the detail time kinetics analyses and by

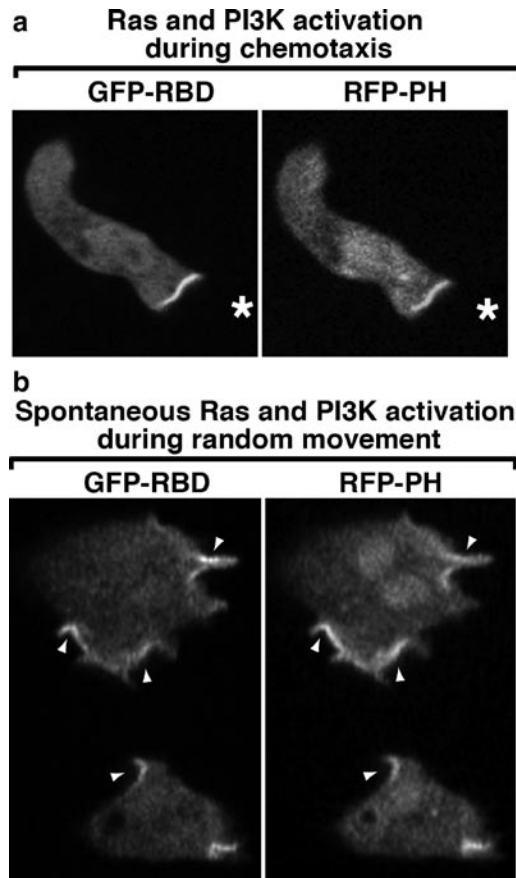


Fig. 1. Analyses of spatio-Ras activation during chemotaxis and random movement. (**a**, **b**) Fluorescent images of GFP-RBD and RFP-PH_{crac} in a chemotaxing (**a**) or randomly moving (**b**) wild-type cell. The Ras activation and PIP₃ accumulation site in a chemotaxing wild-type cell are imaged by GFP-RBD and RFP-PH_{crac}, respectively. An asterisk indicates the direction of chemoattractant source (**a**). Simultaneous imaging reveals that spontaneous Ras and PI3K activation occurs at the same sites (**b**) during random movement. The arrows indicate the Ras and PI3K activation sites. Ras and PI3K activation occur at the same sites and they are synchronized.

using *Dictyostelium* mutant cells that elevate or decreased Ras and Rap activation, such as RasGEF null cells and RapGAP null cells (10, 21, 25, 26). Rap1 was thought to regulate cell adhesion and the leading edge function of moving cells, although there was no direct evidence of a regulatory role for Rap1. With the active-Rap1 detection system described in this chapter, we found that Rap1 is dynamically activated in response to chemoattractant stimulation at the cell cortex with a peak at 5–10 s. Although the total Rap1 protein localizes predominantly on membrane vesicles and the plasma membrane, the activation of Rap1 exclusively occurs at the leading edge in the chemotaxing cells. The spatial distribution of GTP-Rap1 at the leading edge extends more

laterally than that of GTP-Ras, and the kinetics of activation of Rap1 are slower than those of Ras (25). Furthermore, simultaneous imaging analysis, described in the chapter, enables us to distinguish the dynamics of RapGAP1 localization and GTP-Rap1 localization (26). The method described in the chapter led to uncovering new and unexpected mechanisms by which Rap1 controls cytoskeletal reorganization.

In order to detect Ras proteins and small GTPases activation, several methods were developed. A decade ago, the most exclusively used method for assessing Ras activation was to measure the Ras-bound GTP/GDP ratio in $^{32}\text{P}_i$ -radiolabeled cells. Although this method is quantitative, the signal-to-noise ratio tends to be high, resulting in low sensitivity. As millicuries of $^{32}\text{P}_i$ are required, these experiments are not as simple and rapid as nonradioactive experiments, and in some cases radiolabeling itself affects cellular responses. The second generation of Ras assay, which utilizes RBD as a Ras probe, was then developed (27–29). This assay exploits the high-affinity interaction of RBD-Raf1 to GTP-Ras, which is >1,000-fold higher than to GDP-Ras. In the assay, the RBD of Raf1 or RalGDS is fused to GST, and the GST-RBDs are attached to GSH-sepharose beads. The GST-RBD beads are incubated with a cell lysate to pull down active GTP-Ras proteins. This GST-RBD pulldown assay became a major, powerful method to assess Ras and Rap1 activation in the total cell lysate (Fig. 2).

Although the GST-RBD pulldown assay is useful for detecting total Ras activation in the cell lysate, there was no method to detect Ras activation in live cells until recently. In order to detect Ras protein activation in vivo, the GFP-RBD translocation assay and the FRET (fluorescence resonance energy transfer)-based assay have been developed (21, 30–32). Each assay system has its advantages and disadvantages. For information about the FRET-based assay, the reader is directed to references (31) and (32). In this chapter, we will focus on the GFP-RBD translocation assay, in which GFP-RBD accumulation to the plasma membrane or cellular organelles is used as an indicator of Ras activation site(s). Because of its simple readout and the lack of necessity for special equipment, the system is very powerful and can monitor Ras activation with high spatial and time resolution if one has a high-speed camera and confocal or TIRF microscope (Figs. 1 and 2). Strikingly, this is the only system that can detect endogenous Ras proteins' activation in the live cells thus far. However a challenging issue is that the activation of endogenous Ras in mammalian cells is generally not strong enough to visualize GFP-RBD translocation. In many cases, mammalian cell systems require overexpression of exogenous wild-type or mutant Ras. We found that *Dictyostelium* is ideal for studying endogenous Ras and Rap1 signaling because they are robustly activated in this model system. Taking advantage of this, we have studied the role of endogenous

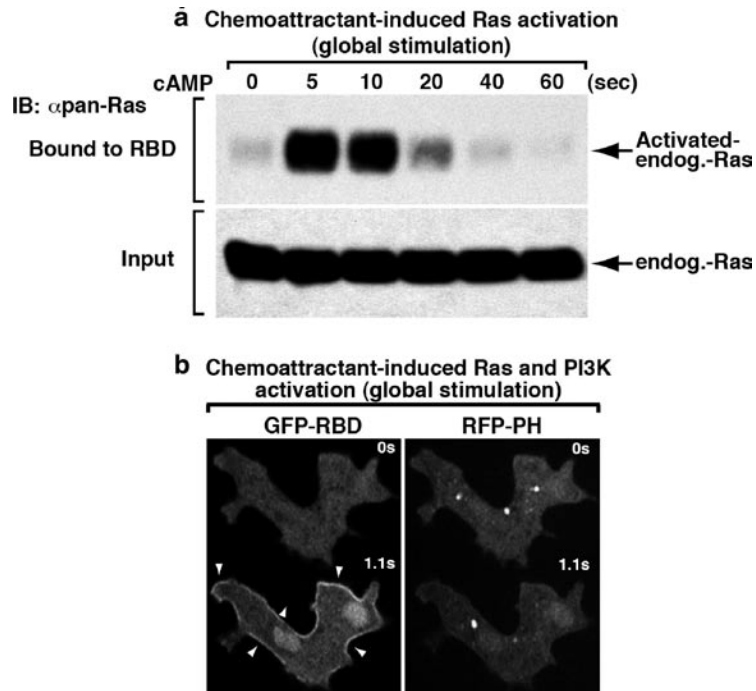


Fig. 2. Ras activation kinetics in response to chemoattractant. (a) GST-RBD-Raf1 pulldown assay shows that endogenous Ras activation occurs rapidly and transiently in response to chemoattractant stimulation. (b) Fluorescent images of GFP-RBD-Raf1 and RFP-PH in wild-type cells before and 1.1 s after the chemoattractant stimulation. The simultaneous imaging reveals that chemoattractant-induced Ras activation occurs prior to PI (3, 4, 5) P3 accumulation at the plasma membrane. The arrows indicate the Ras and PI3K activation sites. The high time resolution (180 ms/frame) also reveals that Ras activation is not synchronized with PI3K activity at the initial phase (~2 s) of chemoattractant signaling.

Ras proteins during random movement and came across a surprising finding. In the absence of chemoattractant and nutrients, both Ras and PI3K activation occurs at the same sites of new pseudopod formation, which provides a critical driving force for random cellular movement. Concomitant Ras and PI3K activation also occurs at the poles of dividing cells, revealing a new insight into the role of spatially restricted Ras activation in cytokinesis (2, 10). *Dictyostelium* is also an outstanding experimental system in which gene knockouts and gene replacements can be readily made; therefore, one can examine Ras activation dynamics in a variety of mutant strains, such as PI3K, RasGEF, and Rap-GAP null cells (10, 21, 25, 26).

Simultaneous imaging of two different molecules provides an opportunity to more precisely determine the order of translocation and activation events (33). Simultaneous time-lapse imaging has been used in several biology fields, such as for the visualization of ions and pH, and this approach is gaining increasing attention and

necessity in the field of chemotaxis. We have applied the simultaneous imaging technology to investigate the interrelationships of Ras protein activation and other molecular events. The method allowed us to uncover the dynamic feedback activation of Ras and PI3K, which is essential for random movement; in response to chemoattractant, Ras activation precedes PI3K activation, while Ras and PI3K activation is synchronized during random movement (**Figs. 1 and 2**)(10). We have found that Rap1 activation is ingeniously regulated by the subcellular localization of Rap1GAP. In response to chemoattractant, there is slightly delayed Rap1GAP translocation compare to Rap1 activation. In the chemotaxing cells, Rap1GAP is found at the inner part of the cell cortex with a partial overlap with the Rap1-activated region at the leading edge. Our simultaneous analyses demonstrate that this Rap1GAP subcellular localization enables cells to tune the spatially and temporally limiting Rap1 activity (26). We hope that the methods described here will benefit and help to elucidate the mechanism for chemotaxis and random movement.

2. Materials

2.1. Buffers and Materials for GST-RBD Preparation

1. BL21 (DE3) *Escherichia coli* competent cells (Stratagene). Store at -80°C .
2. LB Broth: 10 g NaCl, 10 g tryptone, 5 g yeast extract in 1 l of pH 7.0 adjusted with NaOH. Autoclave and store at room temperature. Stable for 1 year.
3. 10 mg/ml filter-sterilized ampicillin. Store at -20°C . Stable for 1 year.
4. 4 $\times$ SDS Sample buffer (4 $\times$ SB): 200 mM Tris-HCl of pH 6.5, 8% SDS, 0.4% bromophenol blue, 40% glycerol. Store at room temperature. Stable for 3 year.
5. 1 M IPTG, store at -20°C . Stable for 3 year.
6. Suspension buffer: 150 mM NaCl, 40 mM Hepes of pH 7.4, 10% glycerol, 1 mM DTT, 1 mM PMSF. Store the buffer without DTT and PMSF at 4°C . Stable for 3 months. Add DTT and PMSF before use.
7. GSH-sepharose 4B (Amersham).
8. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4 , 4.3 mM Na_2HPO_4 , pH 7.4. Store at 4°C . Stable for 1 year.
9. GST-RBD-Raf1 or GST-RBD-RalGDS expression vector for *E. coli*(21, 25, 27–29).
10. 10% NP-40 prepared with distilled water. Store at room temperature. Stable for 3 months.

2.2. Cell Culture and GST-Pull Down Assay

Detailed description of buffers and equipment for *Dictyostelium* preparation is described in (13).

1. Lysis buffer B (2×): 1% NP-40, 300 mM NaCl, 80 mM Hepes of pH 7.4, 40 mM MgCl₂, 20% glycerol, 2 mM DTT, 4 μg/ml aprotinin, 1 mg/ml leupeptin. Store the buffer without DTT, PMSF, aprotinin, and leupeptin at 4°C. Stable for 3 months.
2. Washing buffer: 0.5% NP-40, 150 mM NaCl, 40 mM Hepes of pH 7.4, 20 mM MgCl₂, 10% glycerol, 1 mM DTT. Store the buffer without DTT and MgCl₂ at 4°C. Stable for 3 months.
3. Antibodies: mouse monoclonal anti-Flag antibody M2 (Sigma), anti-pan Ras antibody Ras10 (EMD Bioscience).
4. 30 mM cAMP, pH 6.1. Dissolve cAMP in the Na/K buffer and adjust pH to 6.1. Store in aliquots at −20°C. Stable for 1 year. Use Na/K buffer for making dilutions.
5. 100 mg/ml bovine serum albumin (BSA; Sigma, fraction V) dissolved in PBS. Store at 4°C. Stable for 1 year.

2.3. Time-Lapse and Simultaneous Imaging

1. A confocal microscope. We used Leica inverted DMIRE2 microscope with a 63 × /1.4 objective using an ORCA-ER camera (Hamamatsu). The microscope requires filter sets for the relevant wavelengths and either a 63 or 100× oil immersion objective lens.
2. CCD camera. We used EM-CCD or ORCA-ER, Hamamatsu Photonics.
3. Computer with software for controlling the CCD camera and recording fluorescence images, such as Image J (NIH, Bethesda, MD), Metamorph (Molecular Devices Corporation, Downingtown, PA), or Simple PCI (Hamamatsu, Japan).
4. Cells stably expressing GFP-RBD-Raf1 for Ras assay, GFP-RBD-RalGDS for Rap1 assay.
5. Dual-View OI-11-EM (Mag Biosystem) equipped EM-CCD camera (Hamamatsu).

3. Methods

3.1. Biochemical Analysis of Ras Activation

3.1.1. Preparation of GST-RBD from *E. coli*

1. Transform *E. coli* with GST-RBD expression plasmid (see Note 1).
2. Pick up a single colony and grow in 20 ml LB media with appropriate antibiotics at 37°C by shaking culture (200 rpm) (see Note 2).
3. Transfer overnight *E. coli* culture to 400 ml LB without antibiotics and grow *E. coli* at 37°C by shaking culture (200 rpm) until OD600 becomes 0.5–1.0 at 37°C (see Note 3).

4. Add 125 μ l of 1 M IPTG and keep the shaking culture at 37°C for an additional 1 h (*see Note 4*).
5. Harvest cells by centrifuging 3,000 $\times g$ for 8 min at 4°C.
6. Resuspend the cells with 30 ml Suspension buffer and sonicate for 5 min (*see Note 5*).
7. Add and mix 3 ml of 10% NP-40 to the sample (*see Note 6*).
8. Centrifuge at 10,000 $\times g$ for 20 min at 4°C (*see Note 7*).
9. Transfer the supernatant (*see Note 8*).
10. Filtrate the supernatant with 0.45- μ m filter attached to 30–50 ml syringe (*see Note 9*).
11. Transfer the filtrated lysate to 50-ml tube.
12. Add 700 μ l of GSH–sepharose and rotate for 1 h at 4°C (*see Note 10*).
13. Spin down the sepharose by centrifuging at 500 $\times g$ for 2 min at 4°C.
14. Aspirate supernatant and add ice-cold washing buffer 40 ml. Upside down ten times.
15. Repeat centrifuge and wash totally five times.
16. Add 45 μ l ice-cold 40% Glycerol/PBS (*see Note 11*).
17. Centrifuge and aspirate the supernatant. Store at –20°C (*see Note 12*).
18. Check the GST-RBD production by SDS–PAGE and CBB staining (*see Note 13*).

*3.1.2. GST-Pull Down
Assay for Chemoattractant-
Induced
Ras Activation*

There are several protocols for developing *Dictyostelium* cells (*13, 35*). We have applied the Firtel laboratory method (details in *refs. 13*) described later.

1. Harvest log-phase vegetative cells by centrifuging 5 min at 400 $\times g$.
2. Wash cells twice by centrifuging 5 min at 400 $\times g$ in 40 ml of 12 mM Na/K phosphate buffer.
3. Resuspend the cells at 5 $\times 10^6$ cells/ml in 10 ml of Na/K phosphate buffer.
4. Pulsed the cells for 6 h with 30 nM cAMP every 6 min (*see Note 14*).
5. Treat the cells with 1 ml of 200 mM caffeine for 20 min (*see Note 15*).
6. Collected by centrifugation and resuspended at a density of 2 $\times 10^7$ cells/ml with 2 mM caffeine containing Na/K buffer (*see Note 16*).
7. Aliquot 500 μ l each to 1.5-ml epitube.
8. Add 50 μ l of 100 μ M cAMP (*see Note 17*).

9. Add 550 μ l of ice-cold 2 $\times$ lysis buffer (*see Note 18*).
10. Take time course, for example 0, 5, 10, 20, 40 s (*see Note 19*).
11. Centrifuge 10 min at 15,000 $\times g$ at 4°C (*see Note 20*).
12. Prepare GST-RBD beads (Use GST-RBD-Raf1 for Ras assay, GST-RBD-RalGDS for Rap1 assay): 10 mg GST-RBD per one sample (*see Note 21*).
13. Wash GST-RBD beads twice with 1 $\times$ lysis buffer and suspend in 1 mg/ml BSA containing lysis buffer (*see Note 22*).
14. Take out 60 ml of supernatant and mix with 20 μ l of 4 $\times$ SB for Western blotting (total cell lysate).
15. Transfer 900 μ l of the supernatant to the epitube containing the GST-RBD/BSA mixture.
16. Rotate for 30 min at 4°C (*see Note 23*).
17. Centrifuge for 1 min at 2,000 $\times g$ at 4°C.
18. Aspirate supernatant carefully and add 1 ml wash buffer. Repeat washing again.
19. Add 4 $\times$ SB 20 μ l to the beads (*see Note 24*).
20. Analyze the input and the pull-down product by Western blotting with anti-Ras, anti-Rap1, or appropriate antibody (*see Note 25*).

3.2. Analyzing Ras and Rap1 Activation by Time-Lapse Imaging

3.2.1. Ras and Rap1 Activation in Response to Global Stimulation

Analyzing Ras and Rap1 activation in response to uniform chemoattractant “global stimulation” allows the determination of whether cells have machinery to transmit chemoattractant to Ras and Rap1 activation and adapt to chemoattractant at/above the level of Ras and Rap1 (**Fig. 2**).

1. Prepare the cells expressing GFP-RBD by following **Subheading 3.1.2, steps 1–4**. Use GFP-RBD-Raf1 for Ras assay, and GFP-RBD-RalGDS for Rap1 assay (*see Note 26*).
2. Fill a chambered coverslip with 12 mM Na/K phosphate buffer and add 75 ml of pulsed cells ($\sim 4 \times 10^6$ cells).
3. Allow cells to adhere for 20 min.
4. Place the chamber on the inverted confocal microscope, focus on a cell of interest (*see Note 27*).
5. Focus on a cell of interest, and begin capturing 63 $\times$ or 100 $\times$ images every 0.2 s using imaging software and a CCD camera (*see Note 28*).
6. Use Image J, Metamorph, or Simple PCI software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for viewing and further analysis.

3.2.2. Ras and Rap1 Activation During Chemotaxis

The following protocol is for analyzing Ras protein activation during *Dictyostelium* chemotaxis up to cAMP gradient (**Fig. 1**).

1. Prepare the cells expressing GFP-RBD by following **Subheading 3.1.2, steps 1–4** (*see Note 26*).
2. Fill a chambered cover slip with 12 mM Na/K phosphate buffer and add 75 μ l of pulsed cells ($\sim 4 \times 10^6$ cells).
3. Allow cells to adhere for 20 min.
4. Place the chamber on the inverted confocal microscope; focus on a cell of interest (*see Note 27*).
5. Focus on a cell of interest and use micromanipulator to position a micropipette containing 10 μ M cAMP.
6. Begin capturing 63 $\times$ or 100 $\times$ images every 3 s using imaging software and a CCD camera (*see Note 29*).
7. Use Image J, Metamorph or Simple PCI software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for viewing and further analysis.

3.2.3. *Ras and Rap1 Activation During Random Movement*

The following protocol is for analyzing Ras and Rap1 protein activation of unstimulated vegetative cells randomly moving in the absence of chemoattractant and nutrients. This assay allows the determination of whether spontaneous Ras signaling pathways are involved in random movement (**Fig. 1**).

1. Prepare 1×10^6 cells expressing GFP-RBD in exponential growth phase in nutrient.
2. Seed cells at the density of 2×10^4 cells/cm² in nutrient medium onto a chambered coverslip (*see Note 30*).
3. Allow cells to adhere for 10 min.
4. Wash cells three times in Na/K buffer starvation buffer by aspirating the medium/buffer and adding buffer (*see Note 31*).
5. Add 3–4 ml of Na/K phosphate buffer to cover the cells and let sit for 0.5–1 h (*see Note 32*).
6. Place the chamber on the inverted confocal microscope; focus on cells of interest.
7. Begin capturing 63 $\times$ or 100 $\times$ images every 3–12 s for 30–60 min using imaging software and a CCD camera (*see Note 33*).

3.3. *Simultaneous Analysis of Ras Activation and Other Signaling Events*

The following protocol is for simultaneous imaging of Ras protein activation and other signaling or cellular events, such as PI3K activation, RapGAP translocation, and F-actin polymerization (**Figs. 1 and 2**).

1. Prepare GFP-RBD and RFP-tagged protein expressing cells according to the purpose of the assay (i.e., chemotaxis, global stimulation, or random motility) (*see Note 34*).
2. Place the chamber containing cells on the inverted confocal microscope with a dual-view-equipped camera (*see Note 35*).

3. Focus a cell of interest (*see* [Note 36](#)).
4. Begin capturing 63× or 100× images using imaging software and a CCD camera (*see* [Note 37](#)).

4. Notes

1. *E. coli* strain is important; it is highly recommendable to use *E. coli*, such as BL21 (Stratagene) that does not have much protease activity. We and other groups have used human Raf1 1–149aa region for GST-RBD-Raf1 ([27](#), [28](#)). Others use amino acids 51–131 of Raf-1 and successfully detect Ras activation ([29](#), [30](#)). It has been reported that A85K-RBD-Raf1 mutation increases affinity to GTP-Ras; the GST-A85K-RBD might be useful for the assay ([34](#)).
2. Use big enough flask for aeration: the capacity should be at least five times more than culture media. Other nutrient-enriched media such as Superbroth can be used.
3. It takes about 2–3 h to reach to the optimum cell density. It is important to get exponentially growing cells. Culture without antibiotics does not reduce GST-RBD expression.
4. No need to culture at room temperature. It is important to check the GST-RBD induction by SDS–PAGE and CBB staining. Take 20 µl of culture before and after IMTP and mix with 4× SDS-Sample Buffer 20 µl for the comparison by SDS-PAGE and CBB staining.
5. 50-ml tube is useful. Place the tube into an ice-filled bucket or a 250-ml beaker to keep the sample cool. Sonicate 10 s and give 10-s interval, which is important to keep the lysate cool.
6. Remove 20 µl of lysate and mix with 20 µl of 4× SB for total lysate.
7. Use appropriate tube that can resist to the high-speed centrifugation.
8. Remove 20 ml of the supernatant and mix with 20 µl of 4× SB for soluble fraction.
9. Two or three filters may be required for clogging the filter.
10. The appropriately prepared lysate should contain GST-RBD much more than the GSH-beads (700 µl)-binding capacity. GSH-beads will be saturated with GST-RBD after the incubation. Thus one may increase GSH-beads twice if need to be. Remove 20 µl of lysate after the incubation and mix with 20 µl of 4× SB for flow through fraction.
11. The purpose is to replace the buffer to the storage buffer. Take 20 µl of beads with cut tip and mix with 4× SB 20 µl.

12. GST-RBD beads in 40% glycerol at -20°C are stable for 6 months.
13. To quantify the purified GST-RBD, make a dilution of BSA or any of quantified protein as control. For example, apply 0.1, 0.33, 1, 3.33, 10 μg to the lanes. Apply 10 μl each of the collected samples from the **steps 4, 7, 9, 12, and 16**.
14. cAMP-dependent Ras activation can be seen after 4 h pulsing.
15. This is optional procedure, and the purpose is to inhibit adenyl cyclase and reduce any basal chemotaxis signaling. The treatment also can be done with 1 mM caffeine for 30 min. We also point out that caffeine potentially inhibits many other cellular activities, including PI3K and TOR kinase activity (36, 37). Thus one should consider the use of caffeine according to the aim of the experiment. Also one can examine the side effect of the caffeine treatment by using ACA null strain.
16. The cell density can be decreased to 5×10^6 cells/ml or possibly more. If caffeine treatment is skipped, use Na/K buffer without caffeine.
17. The procedure is designed to capture the early time point, such as 5 s after the stimulation, as the Ras and Rap1 activation is fast ($\sim 3\text{--}10$ s peaks). It is very important to do this quickly (~ 0.5 s) with three times quick pipeting to mix the cAMP equally.
18. It is very important to do this quickly. Close the lid and upside down the tube two times and put the tube into ice.
19. For the early time point, use two pipettes in both hands; with the left hand add 50 μl cAMP and with the right hand add 550 μl of $2\times$ lysis buffer.
20. It is crucial to keep the samples on ice all the time during the assay.
21. If you have ten samples to be tested, take out 100 μg of GST-RBD beads; then wash in a single tube.
22. To wash the GST-RBD beads, add 1 ml of lysis buffer and upside down five time, spin down the beads by centrifuging $2,000 \times g$ for 1 min. Prepare the beads in 100 μl lysis buffer containing 10 mg/ml BSA per sample. It is important to suspend well to aliquot equally. If you have ten samples to be tested, prepare the 100 μg beads in 1 ml lysis buffer containing 10 mg/ml BSA, then aliquot 100 μl each to new tube with O-cut tips. The final concentration of $\sim 1\text{--}5$ mg/ml BSA must be added in the pulldown assay to avoid non-specific binding of Ras proteins to the GST-RBD beads.
23. The incubation time is very critical. Although the incubation times between 20 and 40 min usually work well, we recommend 30-min incubations for consistent results.

24. Spin-column is useful for the elution. Load all the beads to the spin-column, and centrifuge $2,000 \times g$ for 1 min. Place the spin-column into new epitube and add 40 μ l of 1 $\times$ SB directly to the beads in the cup. Centrifuge to collect the GST-RBD bound Ras fraction.
25. 12–15% SDS–PAGE gel is good for separating Ras proteins. If Flag-Ras proteins will be monitored, use more than 3% skim milk in TBST for first and second antibody to reduce background. For anti-Ras antibody (clone Ras10), 3% BSA or 3% goat serum in TBST works well.
26. Stable expression of GFP-reporters is suitable for the assay, as the expression level of the reporter is moderate, thereby the translocation or the accumulation of the GFP-RBD reveals contrasted signal against the nontranslocated GFP-RBD (background). We have not observed any noticeable inhibitory effect in the cells stably expressing GFP-RBDs.
27. It is extremely important to use confocal microscope, which can detect the translocation very clearly. In other words, it is difficult to capture highly contrasted GFP-RBD translocation with standard nonconfocal microscope. Presumably, this may be due to low amount of GTP-Ras existing compared to activation of other molecules, such as F-actin polymerization and PIP3 production, as Ras positions very upstream of signaling cascade (4).
28. Ras and Rap1 activation occurs at peak around 5 s. The frequency of recorded images should be adjusted accordingly.
29. For long-term recording, exposure times and/or exposure frequency may need to be reduced to avoid photobleaching the cells. Thus, sensitivity of microscope system is important.
30. The low cell density is required, as it provide cells to move without attaching each other, and to facilitate tracking individual cell movements within the field of view over the course of the assay.
31. Add buffer carefully onto an aside corner of the dish, so that the attached cells are not to be disturbed.
32. To avoid extracellular stimuli, random motility is assayed in Na/K buffer. However, after >2 h in Na/K buffer, *Dicystostelium* cells secrete pulse of cAMP. Thus, less than 2 h should elapse from the point of addition of Na/K buffer to the end of analysis. Using null mutant strains that cannot secrete cAMP or a perfusion chamber, such as a Dvorak-Stotler chamber (Nicholson Precision Instruments, Gaithersburg, MD), is another way to ensure that no autonomous chemoattractant signaling is occurring.
33. To capture the spontaneous Ras protein activation from the beginning, the frequency of recorded images should

be adjusted to the fastest setting. The shutter will need to remain permanently open.

34. As long as the detection system allows, one can use any combination of CFP, GFP, YFP, RGP-tagging. To obtain cells expressing two plasmids, one can cotransfect with a single drug selection, such as G418 or hygromycin. We have observed high cotransfection efficiency (>70%). The other way is to use two selection markers, for example, GFP-RBD for G418 selection, and RFP-PH domain for hygromycin selection, by subcloning RFP-PH domain into an expression vector carrying hygromycin-resistant gene cassette. So far, we have used the combinations of GFP-RBD-Raf1 and RFP-PH_{crac}, YFP-RalGDS and RFP-RapGAP, YFP-RalGDS and RFP-Coronin, GFP-RapGap1. These simultaneous imaging provided a new insight of Ras and Rap1 signaling inter-relay otherwise not to be seen (**Figs. 1 and 2**) (*10, 26*).
35. Dual View (MAG BIOSYSTEMS) utilizes a single beam splitter to split the incident beam from the microscope into two independent beams, which enables the simultaneous acquisition of GFP and RFP-signal.
36. It is important to select cells that express GFP and RFP-proteins at comparable level.
37. To capture the spontaneous Ras protein activation from the beginning, the frequency of recorded images should be adjusted to the fastest setting. The shutter will need to remain permanently open.

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Chapter 24

FRAP Analysis of Chemosensory Components of *Dictyostelium*

Carrie A. Elzie and Chris Janetopoulos

Summary

Dictyostelium discoideum is a useful cell model for studying protein–protein interactions and deciphering complex signaling pathways similar to those found in mammalian systems. Many of these interactions were analyzed using classical in vitro biochemical techniques. However, with the accessibility of fluorescently tagged proteins, extensive protein networks are now being mapped out in living cells using a variety of microscopic techniques. One such technique, fluorescent recovery after photobleaching (FRAP), has been used in *Dictyostelium* to investigate a number of cellular processes including actin and cytoskeleton dynamics during chemotaxis and cytokinesis (J. Muscle Res. Cell Motil. 23:639–649, 2002; Biophys. J. 81:2010–2019, 2001; Mol. Biol. Cell 16:4256–4266, 2005), to follow trafficking of proteins to organelles such as the membrane, nucleus, and endoplasmic reticulum (Development 130:797–804, 2003; J. Cell Biol. 154:137–146, 2001), and to understand the role of proteins in cell adhesion during motility and division (Mol. Biol. Cell 18:4074–4084, 2007; J. Cell Sci. 120:4302–4309, 2007). FRAP is a powerful tool that should provide a vast amount of information on the mobility of a number of proteins, not only in *Dictyostelium*, but in many organisms. This study will lay out the methods of conducting FRAP experiments in *Dictyostelium* and discuss the large amount of knowledge which can be gained by adopting this as a common technique.

Key words: *Dictyostelium*, Photobleaching, Fluorescent proteins, FRAP, PTEN, PLC

1. Introduction

1.1. Overview of FRAP

Fluorescence recovery after photobleaching (FRAP) is not a novel technique. In fact, FRAP was first developed in the 1970s as a technique to study the mobility of proteins in living cells (8, 9). Experiments were initially aimed at investigating changes in lateral membrane transport as an indicator or consequence of changes in the physiological state of cells. Early success of FRAP

experiments were limited to determining the rates of diffusion of molecules on or in the cell membrane. However, with the advent of fluorescently tagged proteins, which now allow the visualization of proteins in living cells, and the development of confocal microscopy, the use of FRAP experiments has expanded significantly. Today FRAP is used to investigate a plethora of biologic phenomena including motility, division, adhesion, transcription, signal transduction, and protein trafficking.

During FRAP experiments, fluorescent molecules are irreversibly photobleached in a geographically confined region of interest (ROI) using a tightly focused laser beam (**Fig. 1**). Photobleaching is the irreversible loss of the ability of a fluorophore to fluoresce due to photon-induced chemical damage and covalent

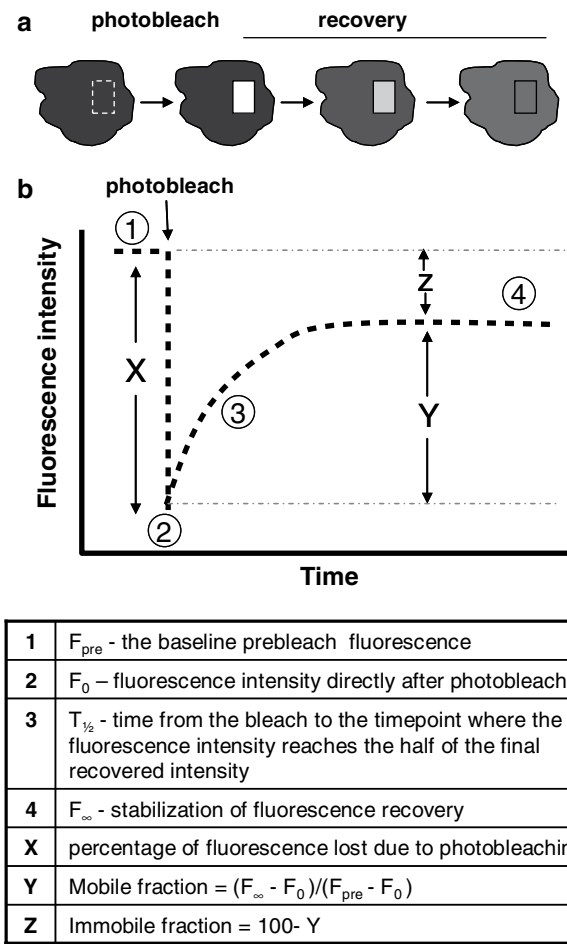


Fig. 1. FRAP experiment. (a) Illustration of a cell undergoing spot photobleaching. The square box represents the region of interest where photobleaching occurs. Over time, the fluorescence in the region of interest recovers. (b) A characteristic recovery curve of fluorescent molecules within the region of interest. Graphing the prebleach intensity and the resulting recovery establishes the mobile fraction within that region. These numbers can all then be used to determine the half-time of recovery, as well as, the diffusion coefficient (see text for specific calculations).

modification. Some fluorophores bleach after only emitting a few photons, while others can undergo many millions of cycles before being covalently modified. Normally the fluorophore is photobleached at a wavelength that is not otherwise absorbed by cellular components, and therefore, should not damage other parts of the cell or specimen (8). The ROI can be in the form of a spot, multiple spots, or a specific area within the cell such as the cytoplasm, cell membrane, or whole organelles. Recovery of fluorescence can come only from molecules that move into the bleached area from outside and replace the bleached molecules within this area. The rate of fluorescent recovery over time in that specific area can be measured and these measurements can be used to calculate the mobility of a specific molecule/protein.

The recovery process is dependent on the rates of diffusion and/or the transport through the cellular milieu. Barriers to diffusion can also be identified and analyzed and assessed using FRAP. The mobility of a molecule can be influenced by binding interactions to proteins, cell membranes, organelles or other changes that affect the local viscosity of the environment in which the molecule resides. Therefore, through careful data analysis, much information can be gained from FRAP including:

- Mobility of a protein/molecule – the percentage of mobile vs. immobile populations
- Recovery rates – how quickly the tagged protein/molecule moves within the cell
- Type of transport – active versus diffusive, random diffusion versus uniform directed flow
- Diffusion constants

1.2. Applications in *Dictyostelium*

Although many of the signaling networks in *Dictyostelium* are very similar to those in mammalian cells, *Dictyostelium* also provides unique differences that researchers can exploit. For instance, signaling and transport of molecules within *Dictyostelium*, as well as basic cellular processes such as phagocytosis, motility and division, often occur on a much faster time scale than in mammalian cells. These processes are easily induced during microscopic observation and allow for fast data acquisition and the ability to carry out more experiments in a shorter amount of time. These characteristics are very useful, but can sometimes be challenging during FRAP experiments. The value of using *Dictyostelium* will be discussed in the following subheaders, while the challenges will be discussed in **Subheading 4**.

1.2.1. Diffusion of Molecules and the Role of the Cytoskeleton

The importance of the simple kinetics of molecular diffusion within cells and the factors which might alter these kinetics are often overlooked in research. However, FRAP experiments have illuminated the significance of kinetics of molecules as they relate to changes in cell shape, developmental stage, cell cycle progression,

and cellular environment. Early on, Potma et al. investigated many of these characteristics in *Dictyostelium* using the green fluorescent protein (GFP) (2). GFP when expressed alone had a 3.6-fold reduction in mobility within *Dictyostelium* as compared to its diffusion in other simple aqueous solutions. The filamentous structures of the cytoskeleton, collisions with macromolecular solutes, and confined motional freedom due to microcompartments within the cell were all likely contributors to this reduction in mobility. In fact, it was shown that the actin cytoskeleton alone accounted for 53% of the restrained molecular diffusion of GFP (2). Thus, changes in the cytoskeleton have profound effects on the diffusion of molecules within the cell and should be taken into account when conducting FRAP experiments. Additionally, cytoplasmic changes that in turn affect the meshwork of actin should also be taken into consideration. For instance, diffusion of GFP was faster in polarized cells than nonpolarized cells. Specific differences in mobility have been noted in the fronts versus the backs of polarized cells (2). Similarly, differences at the cleavage furrow compared with the poles of a dividing cell have also been reported (10). Osmotic properties of the medium have also elicited differences in molecular diffusion, as cells placed in a hypertonic medium showed a decrease in GFP diffusion (2).

Although a significant amount of knowledge in *Dictyostelium* has been gained using GFP alone, the use of FRAP to determine the diffusion of specific proteins in *Dictyostelium* has been somewhat underutilized, especially considering the high number of fluorescently tagged proteins available. Additionally, it is possible to examine the involvement of binding interactions of a protein (*see Note 1*) through comparisons of a GFP-tagged protein to GFP alone. When the FRAP recovery of a GFP-tagged protein is slower than that of GFP alone, binding is implicated and the degree of slowdown of recovery is a measure of the strength of binding. However, an important caveat is that FRAP recovery rates from diffusion are weakly dependent on protein mass (11).

1.2.2. Membrane FRAP

FRAP is a powerful tool for investigating transmembrane proteins or proteins associated with the cell membrane. For instance, FRAP of molecules on the cell membrane can provide information about the molecules' size, environment, and participation in intermolecular interactions, including ligand-driven associations (12). FRAP of transmembrane proteins (such as serpentine receptors) can occur only by the lateral diffusion or directed motion of unbleached proteins in the membrane. Conversely, proteins which interact only with the inner membrane leaflet (G-proteins, PTEN, etc.) will typically have a cytoplasmic population as well. These proteins can recover from FRAP by both lateral diffusion in the membrane and also by exchange with cytoplasmic stores. Therefore, the recovery time after FRAP of transmembrane proteins (lateral diffusion only) is proportional to the area illuminated by the laser beam. Whereas the recovery of membrane-associated

proteins will also include the chemical relaxation time, which is constant regardless of membrane area and thus beam size (13).

Thus, one can determine if a protein is recovering from lateral exchange versus exchange plus diffusion by varying the size of the bleached region of interest on the membrane (14). Recovery rates will be faster for a smaller region of interest rather than a larger one, when analyzing FRAP of transmembrane proteins. On the contrary, recovery rate should not be affected by alterations in bleach spot size for proteins which are undergoing active exchange with the membrane. Additionally, recovery from lateral diffusion only will typically be slower than dynamic exchange. For example, the half-time of recovery of the cAMP receptor cAR1 is about 17 s (data not shown), while the recovery of cortexillin is around 3 s (10). Lateral diffusion versus dynamic exchange can be further analyzed through kymography. A kymograph (also called a time space plot) measures velocities of moving structures in an image time series. Thus, a kymograph of the membrane after FRAP will appear differently from a protein like cAR1 which undergoes only lateral diffusion when compared to PTEN, which is involved in dynamic exchange (Fig. 2). As both these are major

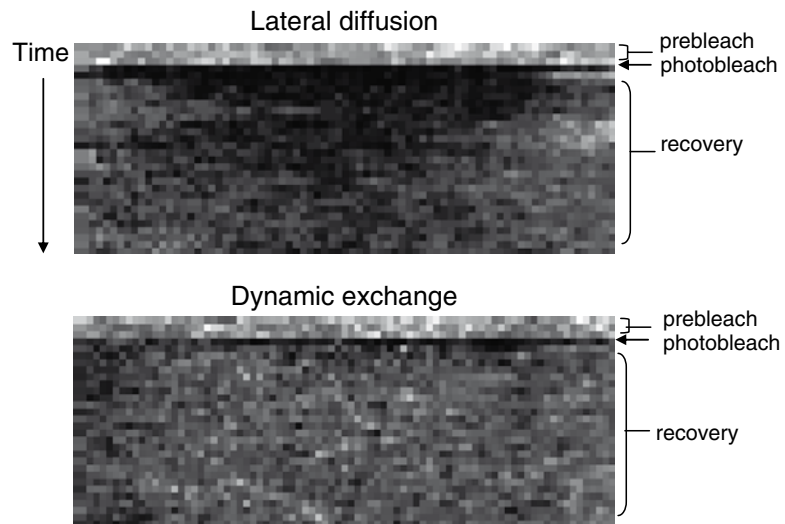


Fig. 2. Characteristic FRAP kymographs from proteins undergoing either lateral diffusion or dynamic exchange to the membrane. For each frame of a time series, gray values along a line type from the region of interest on the membrane were collected. From these lines of gray values, a new image (the kymograph or time-space plot) was assembled with *black boxes* representing low fluorescence and white areas being maximal fluorescence. The line read from the first frame of the time series is put down as the first line in the kymograph, the line from the second frame is the second line of the kymograph, and so forth. In this way, the y axis of the kymograph becomes a time axis and the unit is the time interval of the sequence. The x axis is the distance along the line ROI and the unit is the pixel size of your sequence. Notice that the recovery (*light boxes*) from lateral diffusion of cAR1 occurs first from the sides of the kymograph, then with time, fills in the *middle*. However, dynamic exchange of PTEN recovers uniformly across the region of interest with recovery occurring immediately from the center and from the sides.

players involved in chemotaxis, it would be interesting to investigate whether the recovery rates would differ for these proteins, as well as others, in the presence of cAMP.

1.2.3. Selective FRAP

With advanced software technology and precise mechanical control of the laser spot, photobleached areas are no longer limited to regions of interest in the shapes of circles or squares. Today, many confocal microscopes have an ROI selection tool which allows for bleaching of regions within the cell of varying sizes and shapes. Therefore, entire subcellular compartments can be bleached and their recovery analyzed. For instance, organelles such as the nucleus, endoplasmic reticulum, Golgi apparatus, and mitochondria can now be subjected to FRAP analysis (*see Note 2*). Data from this type of selective photobleaching can be combined with kinetic analysis to determine the rate of cycling between two compartments. Exchange rates between the Golgi and endoplasmic reticulum have already been measured in mammalian cells (*15*). In general, compartmental exchange can occur via diffusion, exchange, vesicular transport or any combination of them.

The newly developed ability to now FRAP complex structures has opened up a new avenue of research for understanding the mechanisms which control protein trafficking through cellular compartments. For example, in *Dictyostelium*, the kinetics of nuclear export of the protein Dd-STATc have been investigated using FRAP of the cell nucleus. Factors that regulate nuclear export were also explored using DIF, a differentiation-inducing factor, and further demonstrated how external factors could in turn regulate transport of proteins within the cell (*4*).

1.2.4. Combining Molecular Techniques with FRAP

FRAP alone as a technique is exciting, but combining it with molecular biology tools makes it a very powerful technique. The recovery of proteins in the presence of various inhibitors of cellular processes can provide information on the regulatory mechanisms of diffusion, recruitment from cytoplasmic pools, or transport of that protein. For example, the inhibition of actin cytoskeletal dynamics in *Dictyostelium* was validated when the diffusion rate of GFP increased significantly after cells were treated with the actin inhibitor, Latrunculin A (*2*). The inhibitor Nocodazole could be used to investigate the role of microtubules, while other drugs such as LY294002 or Wortmannin could help characterize the participation of signaling molecules like PI3-kinase in regulating the mobility of other signaling molecules and effector proteins.

Another utility of FRAP is the comparison of mutant cell lines to wild type. In one case, investigators who varied the levels of talinA in cells affected the dynamics of membrane-associated myosin VII (*6*). In another example, the kinetics of actin crosslinkers were shown to be different in myosin II null cells compared to wild type (*10*). Conversely, the absence of differences in recovery

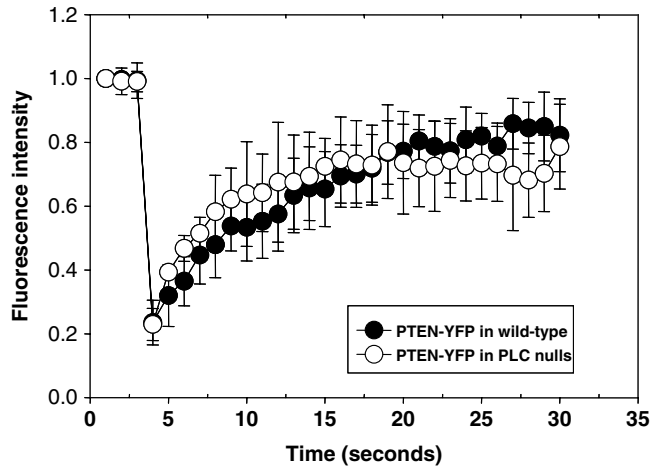


Fig. 3. FRAP recovery of PTEN on the membrane of wild-type cells and PLC null cells. FRAP analyzes of plasma membrane PTEN-YFP expressed in wild-type (AX3) and PLC null cells. No significant difference in recovery was seen between the cell types. For each cell line, the halftime of PTEN-YFP was 2.3 s and the mobile fraction was 77%. Data were collected over two separate days with a total of 18 cells.

rates in mutant cells can also be telling. For instance, we have found that the recovery of PTEN is virtually identical in wild-type and phospholipase C (PLC) null cells (**Fig. 3**), suggesting that the loss of PLC activity in cells has no detectable effect on the basal rate of PTEN membrane association.

The effect of changes in protein structure, sequence, or activity can also be investigated using FRAP through truncation mutants or point mutations. By performing FRAP experiments on truncation mutants, Fukuzawa et al. were able to identify the sequence required for nuclear efflux of the protein Dd-STATc (**4**). The removal of key threonine residues on myosin II completely impaired its recovery after photobleaching, suggesting the importance of phosphorylation in the dynamic turnover of this motor protein (**5**).

2. Materials

1. HL5 growth medium: 0.5% (w/v) proteose peptone, 0.5% (w/v) thiotone E peptone, 55.5 mM glucose, 0.5% (w/v) yeast extract, 1.3 mM Na_2HPO_4 and 2.57 mM KH_2PO_4 . Bring to a volume of 1 l. Adjust pH with HCl to pH 6.4–6.6. Autoclave to sterilize.
2. Developmental buffer (DB): 5 mM Na_2HPO_4 , 5 mM KH_2PO_4 , 1 mM CaCl_2 , and 2 mM MgCl_2 . Prepare the phosphate solution

as 25 mM (5×), adjust the pH to 6.5, and autoclave. Make 10× CaCl₂ (10 mM) and MgCl₂ (20 mM) solutions each separately and autoclave. To make 1 l of DB, mix 600 ml of distilled, autoclaved water with 200 ml of 5× phosphate solution and 100 ml of 10× CaCl₂ and 10× MgCl₂ solution, respectively.

3. Glass bottom culture dishes: 35-mm glass bottom dishes No. 1, uncoated and γ -irradiated (MatTek Corporation).
4. Cells: AX3 wild-type and HD1.19 PLC null cells (Dicty-Base Stock Center <http://www.dictybase.org/StockCenter/OrderInfo.html>).
5. Constructs: PTEN-YFP and cAR1-GFP plasmids with G418 resistance (Devreotes lab).
6. H-50 transformation buffer: 20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM MgSO₄, 5 mM NaHCO₃ and 1.3 mM NaH₂PO₄ in 1 l of distilled water. Adjust pH to 7.0 with HCl or NaOH as appropriate. Autoclave and store cold or frozen.
7. G418: sulfate used at 20 μ g/ml in the HL5 growth medium of the transformants (Invitrogen).
8. cAMP: 50 nM for developing cells.

3. Methods

For **Subheading 3.1–3.7** general methods for performing a FRAP experiment in *Dictyostelium* are described. **Subheading 3.8** includes detailed steps for investigating PTEN recovery on the membrane using FRAP.

3.1. Preparation of Cells

Dictyostelium can be prepared in a variety of ways for FRAP experiments depending on the interest of the investigator. Because axenic media is highly autofluorescent, cells are typically washed and placed in a salt buffer such as development buffer (DB) described earlier. Cells should be allowed to adhere to a glass coverslip at a low density in order to prevent cells from touching one another. This is not a requirement for cytosolic or internal membrane FRAP, but can be critical for plasma membrane FRAP if cells were to contact or overlap each other during the data analysis. Typically it is advisable to have at least one other cell in the image that is not undergoing FRAP to serve as a control for general photobleaching.

3.2. Instruments Used

These techniques require a confocal microscope equipped with a YFP filter and the capability to perform time-lapse imaging in conjugation with photobleaching. In addition, a high numerical

aperture objective is necessary for higher resolution and maximum signal detection. This allows the laser transmission to be reduced in order to minimize non-specific photobleaching of the entire cell. The following protocols are specific to FRAP experiments and are outlined under the assumption that the operator is familiar with the basic operation of the confocal microscope.

3.3. Determining Cell Imaging Parameters for Confocal FRAP

The light intensity and the duration of the bleaching period are adjusted accordingly for the specific fluorophore, developmental stage, and molecule under examination. Investigators should experimentally determine the appropriate duration of irradiation required to reach the extent of photobleaching, appropriate for different specimens (*see* **Notes 3–5**). Typically photobleaching should result in a 60–80% loss of fluorescence intensity in the ROI. To achieve this, one can vary either the laser power strength or the number of iterations that the laser fires. For best results, set the laser at maximum power and then adjust the number of iterations accordingly. This approach minimizes the time required for the actual photobleaching.

The molecule of interest will dictate the objective, filter sets, laser excitation, and zoom required for adequate FRAP. EGFP is commonly used because of its brightness and resistance to photobleaching. Additionally most microscopes are equipped with the proper filter set for GFP. However, other fluorophores can be used successfully with *Dictyostelium* including RFP, YFP, and CFP (data not shown). Other new fluorescent protein variants will certainly become useful for FRAP as microscopes acquire the proper laser lines and filter sets. With repetitive scans some overall photobleaching can occur (*see* **Note 6**). To minimize this effect, it is important to set the laser power and detector gains to the minimum settings required for obtaining images with high signal-to-noise ratios. Additionally the scan speed, pinhole settings, excitation intensity, detector gain, and line averaging should be adjusted for each experimental condition (*see* **Note 7**).

3.4. Selecting the Region of Interest

The confocal microscope software is used to define the bleaching ROI; an often-used geometry is a rectangle, typically with an area of about 100 μm^2 . However, the choice of size will differ depending on the molecule of interest, its distribution in the cell, the recovery speed, the size of the cell, and the method of data analysis. It is important when choosing the size of the ROI to keep in mind that larger areas will typically recover slower than smaller areas. However, if the area is too small, then a significant amount of recovery may occur during the photobleaching phase, thereby skewing your results. Nevertheless, this same phenomenon of masking the recovery can occur with large bleach spots due to the longer time required to bleach the entire area. Therefore, a medium must be established for each molecule examined. However, it may

not be necessary to measure recovery of the *whole* cell and in this case, only the ROI can be measured. This will speed up the data acquisition but will also sacrifice information about overall photobleaching and/or focal plane drifts (14).

For measurements of lateral diffusion on the membrane, it is especially important to use the same bleach size for each experiment to allow for direct comparisons between data sets and to facilitate data analysis. Most software programs will allow the user to either copy and paste the ROI from one cell to another or permit one to type in the exact specifications of the bleach region. Often, the ROI specifications are measured in pixels; therefore, it is imperative to always use the same zoom parameters before photobleaching to maintain consistent bleach data. For selective photobleaching of an entire compartment or organelle, a freehand drawing tool may be required to outline the ROI.

3.5. Determining Proper FRAP Experimental Conditions

Once the bleaching parameters have been established, it is then important to determine the proper temporal settings to capture the recovery. Full recovery will be in the form of a plateau regardless of the percentage of recovery. The speed of recovery will vary depending on the molecule. Examples of recovery times for *Dictyostelium* are shown in **Table 1**. Typically for proteins which recover quickly, images were collected at the speed of the scan time (usually around 1 s). The acquisition speed can be slowed for proteins such as membrane receptors that are less diffusive. Regardless, it is important to minimize the overall nonspecific photobleaching that occurs when collecting multiple images over time. For a 70-s recovery, a reasonable sample rate is 75 images at a rate of 1 image/s (14). To establish a proper baseline for data analysis, it is also important to take a few (2, 3) prebleach images.

3.6. Performing the Actual Experiment

Performing the actual experiment is the easy part once the optimal conditions for imaging, bleaching, and recovery have been established. To obtain accurate data, each experiment should include prebleach images for normalization. Typically three images are acquired prior to photobleaching. Cells should be chosen that have representative expression levels of your molecule of interest. One should obtain an image that has the experimental cell to be photobleached and another cell which is not manipulated. The second cell can serve as a good control for general photobleaching effects. A minimum of ten cells per day should be collected, as it is likely not all will be used because of cell movement and/or the introduction of recovery artifacts. Once the movie has been acquired, it should be examined for artifacts such as changes in focus, cell movement, or significant changes in fluorescent intensities which could confound the data analysis. Examples of these artifacts are shown in **Fig. 4** and should be excluded from further data analysis. Images should be saved in the format specific to the software as well as in a

Table 1
Characteristics of FRAP on published *Dictyostelium* proteins

Protein	Half-time (s)	Diffusion coefficient ($\mu\text{m}^2/\text{s}$)	Immobile fraction (%)	Reference
GFP (cytoplasm)	–	87 ± 2	0.0 ± 0.3	(2)
GFP (cytoplasm) + Latrunculin	–	42 ± 2	3.2 ± 0.3	
GFP (nucleus)	–	22 ± 2	4.0 ± 0.3	
Myosin VII (cytoplasm)	1.0 ± 0.4	–	$\approx 50^a$	(6)
Myosin VII (membrane)	2.8 ± 1.7	–	$\approx 80^a$	
Dynacortin	0.45	–	26	(10)
Fimbrin	0.26	–	33	
Cortexillin I	3.3	–	32	
Myosin II (cortical ring during interphase)	7.28 ± 1.95	–	–	(5)
Myosin II (contractile ring)	7.01 ± 2.62	–	–	
MHCK B (contractile ring)	1.72 ± 0.3	–	–	(3)
MHCK C (contractile ring)	2.32 ± 0.25	–	–	
Actin (mitosis-specific dynamic actin structures)	2.15 ± 0.89	–	–	(7)
Coronin	4.8 ± 1.3	2.2 ± 0.2	–	(1)
cAR1	18 ± 0.41^b	–	$\approx 60^b$	Unpublished ^b
PTEN (membrane)	2.3 ± 1.6	–	23	Reported here

“–”Values not reported

^aAverage interpretations based on graphs reported

^bUnpublished data from the Janetopoulos lab

general format (such as TIFF) that can be used with other software programs. Additionally, it may be useful to save the image with and without the ROI for aiding the analysis.

3.7. Analyzing the Data

Three separate measurements of fluorescent intensity need to be obtained from the data set: pixel values of the ROI, the entire cell, and the background. Depending on the software used, these are typically saved as individual masks which can then be exported to a spreadsheet for further calculations. The larger the background chosen, the more accurate it usually is. However, the background region should be examined for the entire movie to ensure that an artifact or cell doesn't travel through it. Typically the background should not change more than 2–3% during the entire time-lapse series.

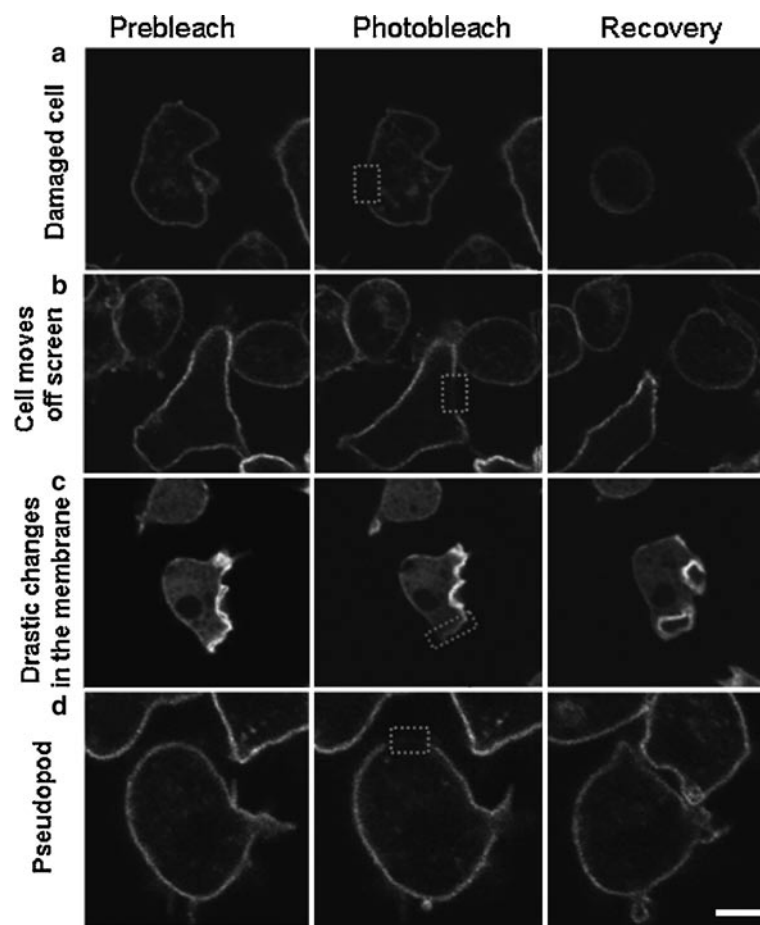


Fig. 4. Cellular artifacts seen with FRAP. Images of cells prior to photobleaching, immediately after, and at the end of the recovery are shown. These cells represent data that would be discarded from analysis. (a) The cell completely rounds up indicating excessive damage or possible death. (b) The cell moved off the screen by the end of the time-lapse acquisition. (c) The FRAPped region on the membrane was hard to distinguish. (d) A pseudopod formed on the membrane where FRAP occurred.

Once these raw values are obtained, the following calculations should be done:

1. Subtract the background fluorescence from the whole cell fluorescence for each frame.
2. Subtract the background fluorescence from the ROI for each frame.
3. Divide the adjusted ROI value (#2) by the adjusted whole cell value (#1) in order to correct for general photobleaching within the cell.
4. Normalize the data to the prebleach intensity by dividing each frame value (#3) by the prebleach intensity of frame 1 and multiplying by 100. This will give you the percentage of initial fluorescence recovered (see example of data from one cell in [Table 2](#)).

Table 2
Example data set from FRAP on PTEN-YFP of one cell

Time (s)	Background	Whole cell	ROI	Step #1	Step #2	Step #3	Step #4
				Whole cell – background	ROI – background	#2/#1	(#3/2.35) × 100
0	183.14	1,004.56	2,115.49	821.42	1,932.35	2.35	100
1	181.69	971.05	2,087.98	789.36	1,906.29	2.41	102.66
2	177.92	939.32	2,031.46	761.4	1,853.54	2.43	103.48
3	169.79	728.15	448.55	558.36	278.76	0.50	21.22
4	173.55	714.7	658.45	541.15	484.9	0.90	38.09
5	175.43	705.82	755.98	530.39	580.55	1.09	46.53
6	171.54	700.87	846.26	529.33	674.72	1.27	54.18
7	174.33	671.91	947.19	497.58	772.86	1.55	66.03
8	175.44	655.2	960.02	479.76	784.58	1.64	69.52
9	168.56	668.03	989.62	499.47	821.06	1.64	69.88
10	173.51	665.53	973.33	492.02	799.82	1.63	69.10
11	171.38	664.92	858.81	493.54	687.43	1.39	59.21
12	179.16	660.32	986.38	481.16	807.22	1.68	71.32
13	178.68	656.7	993.51	478.02	814.83	1.70	72.46
14	181.44	654.89	964.72	473.45	783.28	1.65	70.33
15	185.63	654.35	1,185.08	468.72	999.45	2.13	90.64
16	176.58	658.79	1,189.66	482.21	1,013.08	2.10	89.31
17	186.04	646.84	1,071.14	460.80	885.1	1.92	81.65
18	182.12	645.09	1,145.35	462.97	963.23	2.08	88.44
19	178.58	658.39	1,156.40	479.81	977.82	2.04	86.63
20	179.66	661.27	1,143.36	481.61	963.80	2.00	85.97
21	179.68	647.25	1,049.53	467.57	869.85	1.86	79.08
22	173.48	644.72	1,107.93	471.24	934.45	1.98	84.29
23	177.55	654.29	1,145.78	476.74	968.23	2.03	86.33
24	182.07	642.61	1,141.64	460.54	959.57	2.08	88.57
25	180.52	645.71	1,176.73	465.19	996.21	2.14	91.03
26	175.02	647.05	1,201.81	472.03	1,026.79	2.18	92.47
27	174.68	641.86	1,112.38	467.18	937.7	2.01	85.32
28	176.89	653.04	1,159.17	476.15	982.28	2.06	87.69
29	173.93	651.99	1,182.13	478.06	1,008.2	2.11	89.65

Once calculated, the final numbers can be graphed on any graphing program to determine the percent bleached, the recovery, and the plateau. From this graph, both the mobile and the immobile fractions of the molecule can be determined (*see Fig. 1*). A kymograph can also be made to qualitatively visualize the recovery and to ascertain whether just lateral diffusion is occurring on the membrane or whether there is dynamic exchange (*see Fig. 2*).

The mobile fraction (M_f) is the percentage of molecules which are free to recover from photobleaching during the time course (*Fig. 1*). The fraction of molecules that cannot exchange between bleached and nonbleached regions is called the immobile fraction. The mobile fraction can also be calculated from your data set by using the following equation: $M_f = (F_{\square} - F_0) / (F_{\text{pre}} - F_0)$, where F_{pre} is the prebleach intensity, F_0 is the intensity directly after photobleaching, and F_{∞} is the final postbleach intensity which should be within the plateau. The immobile fraction is then calculated by subtracting the M_f from 100.

The half-time of recovery is the time from the bleach to the time point where the fluorescence intensity reaches the half ($t_{1/2}$) of the final recovered intensity (F_{∞}). The half-time serves as a relevant marker for comparative FRAP analyses, e.g., to measure the mobility of a labeled protein under different physiological conditions. The shorter the half-time, the higher the mobility will be of the observed protein. This allows you to determine the effective diffusion coefficient of the molecule.

Many methods have been described to calculate the diffusion coefficient depending on the type of diffusion occurring and on the nature of the ROI that was photobleached. The two-dimensional diffusion equation by Axelrod et al. is most commonly used for simple diffusion of molecules within the cytoplasm or membrane (8). For examples of other calculations *see ref. 14*. However, caution should be taken when comparing diffusion coefficients between different cell lines or compartments as the diffusion of the same molecule in different compartments may vary significantly due to the architecture of that compartment (16).

3.8. Detailed Methods for Membrane FRAP of PTEN-YFP

1. Transform wild-type AX3 and HD1.19 PLC null cells with PTEN-YFP by electroporation with 5.0 μg of plasmid using established methods (17, 18). G418-resistant clones are selected in 2–3 weeks. Culture cell lines axenically in HL5 medium at 22°C in the presence of G418 to maintain selection of transformants.
2. Prior to FRAP, develop cells by washing them twice with DB buffer followed by 5.5 h of starvation with continuous shaking (19) (*see Note 9*). Pulse cells with 50 nM cAMP every 6 min for the last 4.5 h of starvation.
3. Seed cells in DB on glass bottom dishes and allow them to firmly attach for 15 min prior to FRAP.

4. For these particular experiments, an Olympus FV-1000 confocal microscope and its corresponding software were used. Although this microscope is equipped with a separate laser for imaging and bleaching, the data were collected using a single HeNe laser at 515 nm for both, as this is currently the more accessible approach.
5. Perform the experiments with a 60 $\times$ /1.45 Plan-Apochromat oil objective.
6. For adequate bleaching of PTEN-YFP use a 515-nm laser for ten iterations at 100% power (*see* **Note 3**).
7. Set the pinhole at 1 Airy unit and do not use line averaging. These settings allow you to photobleach approximately 80% of the initial fluorescence without creating any visible signs of damage to the cell.
8. For FRAP of the membrane, use a freehand drawing tool to create the ROI. This allows you to create the appropriate ROI when there are significant curves along the cell perimeter. However, to maintain consistency, you should maintain the maximum width along the membrane constant at 4.5 μ m and the average depth below and above the membrane between 1 and 1.5 μ m depending on how much the cell moved before bleaching.
9. Images should be acquired at the speed of the scan (about 1 s) due to the quick recovery of PTEN. The amount of time taken to bleach the ROI is about 3 s. Full recovery will occur about 9 s after bleaching, so it is possible that some recovery was actually occurring during the 3-s bleach and remains unaccounted for with this method of acquisition.
10. Analyze the recovery over 30 s, which is sufficient to reach a plateau (**Fig. 3**). During this time the average of overall photobleaching in the cells undergoing photobleaching should be calculated (approximately 40–50% for PTEN-YFP cells). This number should be accounted for in the data analysis.
11. Collect three prebleach images of cells for normalization of the data.
12. Collect images of 15–20 cells of varying intensities on three different days.
13. Exclude cells that move too much, have significant changes in the shape of their membrane, or have substantial changes in fluorescent intensity from further data analysis (**Fig. 4**).
14. To analyze PTEN recovery to the plasma membrane, draw an individual line on the membrane (instead of taking a mask of the entire ROI). The entire cell including the membrane should be used for normalization. Choose a background near the cell that is roughly half the size of the cell. Examples of each of these can be seen in **Fig. 5**) All measurements were

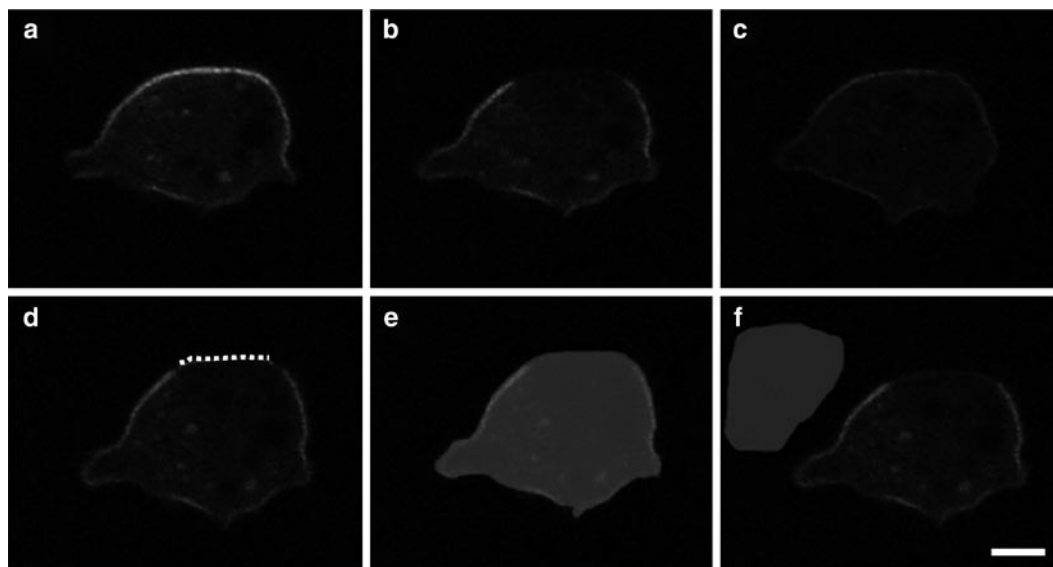


Fig. 5. Examples of masks from the data analysis of PTEN-YFP in AX3 cells. Images of PTEN-YFP expressed in AX3 wild-type cells are shown prior to photobleaching (a), immediately after (b) and at the end of recovery (c). Example masks for analysis are shown for the membrane (dotted line) (d), whole cell (shaded area) (e), and background (shaded area) (f). Bar equals 3 μ m.

obtained were analyzed using Slidebook software, but many other forms of software are available.

15. Graph PTEN recovery using any standard graphing software. We use SigmaPlot software and perform the kymographs using the kymograph plug from ImageJ, which is available to download for free from the NIH.
16. The mobile fraction of PTEN in both wild-type cells and in PLC nulls cell is 77%. This number was generated by using 99.4 as F_{pre} , 23.5 as F_0 and 82.2 as F_{∞} . Thus, the immobile fraction is 23%.

3.9. The Future of FRAP

In the coming years we can look forward to widespread use of confocal FRAP as well as the implementation of new techniques which enhance the imaging and/or data acquisition of these experiments. Already gaining momentum is the use of multicolor FRAP which allows for the simultaneous analysis of two or more fluorescent molecules that have minimal to no spectral overlap (such as eGFP and mCherry). The use of two-colored FRAP could be used to study the dynamics of two proteins in different but overlapping cellular compartments and of two proteins of the same complex. Moreover, mutant proteins could be directly compared with wild-type versions within the same cell (20).

Another exciting technique combines FRAP with total internal reflection fluorescence microscopy. This technique, called total internal reflection interference fringe fluorescence photobleaching

recovery (TIRIF-FPR), has been advantageous for sparsely expressed membrane species or when high expression in the Golgi or ER masks membrane expression. Additionally it can be used to measure cell receptors labeled with fluorescent antibodies or ligands (21). It should also be noted that FRAP can and should be used to complement data generated using single molecule imaging. Single molecule imaging, usually done using total internal reflection microscopy, often suffers from artifacts resulting from the blinking and bleaching of fluorophores. Half times and diffusion rates for molecules that shuttle from one compartment to another should be determined using both technologies.

The recovery of molecules in one plane using confocal microscopy presents unique challenges for structures that are moving and also is confounded by the fact that the profile of the bleach spot in the z direction cannot be determined looking at a single plane. One way to visualize the full recovery is to do a rapid z scan and watch the recovery in three dimensions. Brighter fluorescent proteins, more sensitive cameras, and more precise z stepping will make this standard practice in the near future. We are also experimenting with another solution. We have developed mirrored pyramidal wells that allow the imaging of a cell after photobleaching from multiple perspectives (22). In addition, we can direct the bleaching laser off the side reflections and also bleach the cell in the x and y directions and watch the recovery from multiple perspectives (Fig. 6). This gives unprecedented flexibility for bleaching and will allow the experimenter to visualize the bleached area with much higher spatial resolution.

The benefits of these advanced techniques will be an improved knowledge of protein-binding interactions and trafficking in *Dictyostelium*. This knowledge combined with that gathered in other cell systems will further our understanding of complex signaling networks and their relation to cell behavior.

4. Notes

1. It should be noted that many fluorophores including eCFP, eGFP, and eYFP can undergo light-induced and pH-dependent reversible photobleaching (25). This could potentially generate artifacts during FRAP. If you bleach the whole cell and no recovery occurs, then this can typically be ruled out as an issue.
2. For FRAP experiments on organelles with complex three-dimensional shapes (such as the endoplasmic reticulum (ER)), additional z -planes should probably be imaged. Several papers have discussed ways to incorporate these calculations (26–28).

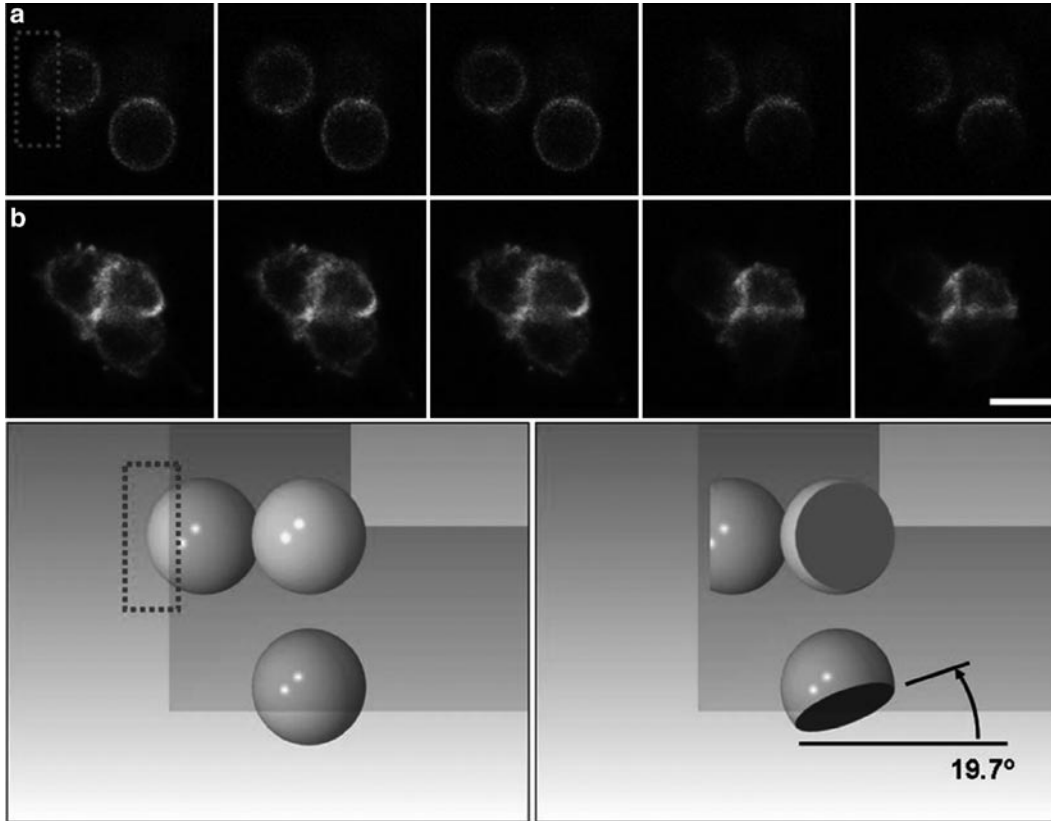


Fig. 6. Fluorescence recovery after photobleaching (FRAP) experiments in mirrored pyramidal wells (MPWs). Shown is a series of two individual cells expressing cAR1-GFP along with their reflections prior to and after selective photobleaching in an MPW. Top panel: Five successive frames of a Latrunculin-treated, immobilized cell (*top row, a*) and an untreated cell (*bottom row, b*), each of which was positioned in the corner of an inverted MPW. In each case, laser scanning of the upper left reflected image leads to selective photobleaching of the dorsal surface of the cell, as can be seen in reflected images in the same frame (bleaching occurs after frame 3 in each series). The angle of the photobleached section is very near the expected angle. *Bottom panel:* A SolidWorks simulation of the photobleach experiment illustrating the cell before bleaching (*left*) and after bleaching (*right*), and the shape of the bleached section of the *sphere* as seen from above and from both reflections. Image is courtesy of Kevin Seale. Bar is 15 μm .

3. Inadequate photobleaching may occur when either the number of laser iterations or the strength of the laser is too low. It is recommended that the laser is set to full power and then alter the number of iterations to avoid general photobleaching and to minimize the amount of time taken to perform the actual photobleaching.
4. If no recovery is observed, then it is possible that the recovery is slow and the time lapse should be extended. When doing long time lapses, it is important to space out the number of images taken in order to avoid excess exposure. Alternatively, a time delay can be set to increase the interval between images. No recovery may also result from a cell being irreversibly damaged or killed.

5. If the recovery is very fast, then part of it may be masked by the acquisition parameters. To correct for this, try using a scope with two separate lasers – one for imaging and one for bleaching. If a separate laser is not available, then try increasing the speed of data acquisition by only collecting data from the ROI versus the entire cell. However, it is important to note that this method sacrifices information about overall photobleaching and/or focal plane drifts which may impact your data. Alternatively, the size of the ROI can be increased.
6. During prebleach and postbleach image acquisition, the lowest possible illumination intensity should be used. This minimizes bleaching of the sample during monitoring. For detection purposes “Gain” and “Offset” should be set to ensure the dynamic range is exploited to the fullest.
7. It is important to be aware of the fact that both temperature and pH can affect diffusion, so these parameters should be kept constant (24).
8. Photobleaching can be damaging to cells; therefore, it is important to make sure that the cell is not too damaged or dead at the end of your time lapse. To do this make sure the cell is still moving throughout the time lapse and hasn’t rounded up (see example Fig. 4a).
9. Highly mobile cells are hard to FRAP and analyze; this is particularly true of cells grown on bacteria and of highly polarized cells that have been developed. To compensate for this, cells are typically only developed for 5.5 h so they are responsive to cAMP, but not quite polarized. However, this may be cell type specific and may not be possible if you are interested in cells characterized by polarized morphology. Actin inhibitors, like Latrunculin, will immobilize cells and make FRAP easier. However, as noted earlier, dissolution of actin can have significant effects on the diffusion of molecules within the cell. One alternative is to compress cells between glass and an agar layer in order to flatten them and to reduce their mobility (1). Another is to mechanically compress them in a device such as a rotocompressor (23).

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Chapter 25

Monitoring Dynamic GPCR Signaling Events Using Fluorescence Microscopy, FRET Imaging, and Single-Molecule Imaging

Xuehua Xu, Joseph A. Brzostowski, and Tian Jin

Summary

How a eukaryotic cell translates a small concentration difference of a chemoattractant across the length of its surface into highly polarized intracellular responses is a fundamental question in chemotaxis. Chemoattractants are detected by G-protein-coupled receptors (GPCRs). Binding of chemoattractants to GPCRs induces the dissociation of heterotrimeric G-proteins into $G\alpha$ and $G\beta\gamma$ subunits, which in turn, activate downstream signaling networks. To fully understand the molecular mechanisms of chemotaxis, it is essential to quantitatively measure the dynamic changes of chemoattractant concentrations around cells, activation of heterotrimeric G-proteins, and the mobility of GPCR and G-protein subunits in the cell membrane. Here, we outline fluorescence imaging methods including Förster resonance energy transfer (FRET) imaging and a single-molecule analysis that allow us to measure the dynamic properties of GPCR signaling in single live cells.

Key words: Confocal fluorescence microscopy, Förster resonance energy transfer, Total internal reflection fluorescence microscopy, Single-molecule imaging, GPCR, Heterotrimeric G-proteins, Spatiotemporal dynamics

1. Introduction

Migration of leukocytes, T cells, and B cells is directed by extracellular diffusible chemicals known as chemokines ([1](#)). These chemokines are detected by G-protein-coupled receptors (GPCRs) on the cell surface, which in turn activate (dissociate) heterotrimeric G-proteins on the inner cell membrane ([1–3](#)). The dissociation of G-proteins into $G\alpha$ and $G\beta\gamma$ subunits triggers multimolecular

signaling networks in the cell leading to directional cell migration (4). To fully understand the molecular mechanisms underlying chemotaxis, it is essential to quantitatively measure spatiotemporal dynamics of GPCR signaling, including the concentration of chemoattractant, the activation of and potential changes in mobility of GPCRs and G-proteins in live cells (5).

The soil amoeba *Dictyostelium discoideum*, like mammalian leukocytes, is able to pursue and ingest bacteria via chemotaxis and phagocytosis, respectively (4, 6). *D. discoideum* has been established as a key model system to reveal the molecular mechanisms of chemotaxis. When starved of growth nutrients, cAMP is secreted by cells and acts as the extracellular chemoattractant to mediate the migration (aggregation) of individual amoeba into an organized multicellular structure. A family of developmentally regulated GPCRs, cAR1-4, detects the extracellular cAMP signal (7). The cAR1 GPCR is the major receptor that regulates robust chemotaxis of *D. discoideum* cells during the aggregation stage (8). The cAR1 receptor couples to the heterotrimeric G-proteins $G\alpha_2/G\beta\gamma$ to mediate the signaling networks that are responsible for cAMP gradient sensing and cell migration (9, 10). In the past 10 years, live cell imaging techniques have been developed to measure dynamics of cAR1-mediated signaling network in live *D. discoideum* cells.

Here, we introduce three imaging methods that have been successfully applied to measure spatiotemporal changes of signaling events in single live cells in real time. First, we describe how to measure the concentration of an applied chemoattractant in live cell experiments. To fully understand how cells migrate in response to temporal and spatial changes of chemoattractants, it is essential to monitor the concentration of chemoattractant that has been applied to the cells in real time. We have developed a method that allows us to visualize and quantitatively measure the spatiotemporal changes of an applied cAMP stimulus around cells by mixing the chemoattractant with a fluorescent dye (10, 11). Using confocal fluorescence microscopy, we can monitor the intensity of fluorescent dye and calculate the cAMP concentration that a cell is exposed to during its chemotactic response. If cells express a fluorescent marker to show intracellular changes in response to cAMP stimuli, such changes can be simultaneously recorded and correlated with changes in chemoattractant concentration.

Second, we present a Förster resonance energy transfer (FRET) imaging method, which has been used to monitor GPCR-mediated dissociation (activation) and reassociation (deactivation) of heterotrimeric G-protein in single live cells (10, 11). Protein/protein interactions cannot be measured by colocalization of the proteins because the limit of resolution of the light microscope using standard techniques is on the order of hundreds of nanometers, and one cannot be certain that two proteins of interest physically interact even

when they appear to be colocalized by fluorescence microscopy. FRET technology breaks this barrier, providing a means to monitor the interaction between proteins at a quantitative level in live cells at distances less than 10 nm. The method can detect changes in the conformation of a protein or the interaction between two proteins that are tagged with a FRET donor/acceptor pair, such as ECFP and EYFP. When the donor and the acceptor fluorophores are fused to a single protein, conformational changes that increase/decrease the distance between the fluorophores can be observed as a decrease/increase in FRET, respectively. Imaging and quantifying FRET can be technically challenging. FRET requires an overlap of the donor emission and acceptor excitation spectra. While this spectral proximity enables FRET to occur, signals generated by bleed through of donor emission into the FRET detection channel and direct excitation of the acceptor by the donor excitation light source must be subtracted from the FRET image. Some of these technical difficulties can be overcome by using a confocal microscope that obtains emission spectra rather than a single intensity measurement obtained through a bandpass emission filter (12). Combined with fast image acquisition, dynamic conformational changes or protein-protein interactions detected by changes in FRET signal are monitored not only spatially but also temporally with a resolution of about 1 s making the technique appropriate for live cell imaging.

Third, cell surface receptors and their effectors diffuse in the cell membrane, and this dynamic aspect is an essential part of receptor signaling. Single-molecule microscopy is capable of monitoring the mobility of individual proteins fused with fluorescence proteins, such as EGFP or EYFP, on the plasma membrane of live cells. Using a total internal reflection fluorescence microscopy (TIR-FM) (13), we have imaged GPCR and G-protein $\beta\gamma$ subunits in the plasma membrane at a single-molecule level in live cells in real time to determine how these proteins diffuse in the plasma membrane. We introduce a single-molecule imaging method that permits one to measure the mobility of GFP (or YFP)-tagged GPCR or G-protein subunits in the plasma membrane of live cells.

2. Materials

2.1. *D. discoideum* Cell Culture

1. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) (Gibco, Invitrogen Life Science) solutions are stored at -20°C .
2. D3-T medium (KD Medical, Columbia, MD). Penicillin and streptomycin are added to D3-T medium at final concentrations of 30 U/mL and 30 $\mu\text{g/mL}$, respectively.

3. 10-cm round tissue culture dishes are used for stationary growth (Falcon, Franklin Lakes, NJ).
4. 125- and 250-mL plastic Erlenmeyer flasks (Corning, Corning, NY) are used for shaking culture.

2.2. *D. discoideum* Development

1. Development Buffer (DB): 1.34 g Na_2HPO_4 , 0.68 g KH_2PO_4 /L with 0.2 mM CaCl_2 and 2 mM MgSO_4 .
2. 0.01 M cAMP (Sigma, Steinheim, Germany) in water is stored at -20°C . The final pulse concentration is 100 nM.
3. ChronTrol XT programmable timer (ChronTrol Corp, San Diego, CA).
4. Miniplus 3 peristaltic pump (Gilson, Middletown, WI).
5. Platform rotary shaker with accurate rpm control.
6. 125-mL plastic Erlenmeyer flasks.

2.3. HEK 293 Cell Culture

1. IMEM medium (Cellgro, Mediatech Inc, Manassas, VA).
2. Fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA).
3. Trypsin (Cellgro, Mediatech).
4. L-Glutamine (Gibco, Invitrogen Life Science, Bethesda, MD).
5. Geneticin® (Gibco, Invitrogen Life Science), A stock concentration of active 50 mg/mL (1,000×) is stored at -20°C in aliquots.
6. T25 flask (BD Biosciences, Falcon).
7. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solutions are frozen at -20°C (Gibco, Invitrogen Life Science); 5 mL is added to 0.5 L of IMEM complete culture medium.
8. Cell lines: HEK293 cells transiently expressing CFP, YFP, or Yc6 plasmid which encodes cDNA of CFP-calmodulin-YFP sequence. Cells were starved with IMEM medium without FBS 24 h before experiment.
9. Cell culture incubator, 37°C , 5% CO_2 .

2.4. Dyes for Chemoattractant Imaging and Chemoattractant Delivery

1. Alexa 488, Alexa 594, and LysoTracker Red DND-99 (Invitrogen-Molecular Probes). A 1 $\mu\text{g}/\mu\text{L}$ stock is made in water and stored at -20°C . The working concentration is 0.1 $\mu\text{g}/\mu\text{L}$.
2. Single-well Lab-Tek II coverglass chambers (Nalge Nunc International, Naperville, IL).
3. Femtotip microcapillary pipets (Eppendorf, Germany).
4. FemtoJet microinjector (Eppendorf, Germany).
5. TransferMan NK2 micromanipulator (Eppendorf, Germany).

**2.5. Confocal
Fluorescent
Microscope**

1. LSM 510 META (Carl Zeiss) with either a 40× 1.3 NA Plan-Neofluar or 63× 1.4 NA Apochromat objective lens.
2. LSM 510 META software to process time-lapse fluorescence images, and spectrally resolved images of Lambda Stack using the Linear Unmixing Function of LSM510 META.

**2.6. TIR Fluorescent
Microscope**

1. Olympus microscope IX81 (Olympus America, Inc., Center Valley, PA).
 - (a) Zeiss Plan-Fluar 100× OIL/1.45 N.A.
 - (b) Electron Multiplier CDD camera, Cascade II 512×512 (Roper Scientific).
 - (c) Software, MetaMorph for Windows.
2. VWR microcover glass #1.5, 24 × 50 mm (Fisher).
3. Lab-Tek chambered #1.0 borosilicate cover glass (eight-well chamber).
4. Coplin Jar (Wheaton, No. 900570).
5. Nano Strip (Cyantek Corporation).
6. 200 proof ethanol, USP grade (Warner-Graham).
7. Compressed argon or AccuDuster III (CleanTex).
8. Sylgard 164 Silicone elastomer Part A/B.
9. Slide chamber (Fisher).
10. Roll & Grow™ Plating beads (Bio 101 System).
11. Dumont electronic tweezers, style 2A (Ted Pella).

3. Methods**3.1. Dictyostelium
Growth and
Development**

1. Grow cells to $3\text{--}5 \times 10^6/\text{mL}$ in D3-T medium.
2. Wash cells twice with DB.
3. Resuspend at 2×10^7 cells/mL in DB and transfer to flask.
4. Shake at 120 rpm for 4–5 h.
5. While shaking, use Chronotrol timer and peristaltic pump to rapidly introduce cAMP to 50 nM (i.e., “pulse”) every 6 min to promote development.
6. For microscopy, deposit cells in chambered cover glass slides.

**3.2. Measurements
of Applied
Chemoattractant
Stimulations**

To quantitatively measure an applied cAMP concentration in live cell experiments, Alexa 594 mixed was mixed with cAMP and imaged using a confocal microscope with a Z-axis resolution of 1–2 μm (*see* [Note 1](#)). The intensity change of Alexa 594 and cell

responses are simultaneously recorded in two different channels as a time-lapse movie. The concentrations of cAMP in various regions around the cell can be determined based on the fluorescence intensity of Alexa 594 in each image of the time-lapse experiment. Images of Alexa 594 fluorescence are recorded by excitation with a 543-nm laser line and an emission bandpass filter (580–650 nm). The Z-axis resolution is usually $<2\ \mu\text{m}$ so that unwanted fluorescent signal from other focal planes is eliminated. We have used different procedures to apply several kinds of cAMP stimuli in live cell experiments.

1. *Exposing a cell to a uniform cAMP stimulation.* To generate a uniform stimulation, a 100 μL mixture of cAMP and Alexa 594 (0.1 $\mu\text{g}/\mu\text{L}$) is applied to the cells submerged in 400 μL DB in a four-well chamber. Under these conditions, cells were generally exposed to a uniform cAMP stimulation that was about 2.5-fold of the final cAMP concentration.
2. *Establishing a steady cAMP gradient.* To establish a cAMP gradient, a Femtotip micropipette is filled with 30 μL of solution containing cAMP and Alexa 594 (see [Note 2](#)) and then attached to the FemtoJet microinjector and micromanipulator. The output pressure of the FemtoJet is set at $P_c = 70\ \text{hPa}$ to release a constant and tiny volume of the cAMP/Alexa 594 mixture into a one-well chamber that is filled with 6 mL of DB. Under these conditions, a gradient can be established within 100 μm from the tip of the micropipette and is usually stable for more than 1 h ([Fig. 1](#)).
3. *Suddenly exposing a cell to a cAMP gradient.* Two positions of micropipette are preset with TransferMan NK2 micromanipulator: Pos. 1, close to the cell; Pos. 2, 1,000 μm away from the cells. The micropipette filled with the cAMP/Alexa 594 mixture is connected to the FemtoJet microcapillary pressure supply unit and set at the Pos. 2. To quickly expose a cell to a cAMP gradient, the micropipette is moved to Pos. 1 automatically within 100 μm to the cell in less than a second ([Fig. 1c](#), see [Note 3](#)).
4. *Quickly withdraw a cAMP gradient from a cell.* To withdraw a gradient from a cell, a micropipette is quickly moved to Pos. 2 (more than 1,000 μm) from a cell (Pos. 1) in less than 1 s ([Fig. 1c](#), see [Note 3](#)).

3.3. FRET Time-Lapse Imaging in Live Cells

FRET imaging can monitor dynamic interactions between a FRET donor (ECFP) and an acceptor (EYFP), which are fused to the protein of interest, in live cell experiments. As an example, we show the FRET changes of a fluorescent biosensor for Ca^{2+} , cameleon, using the spectral detector of the Zeiss LSM 510 META in a time-lapse imaging experiment. Cameleon is a chimeric protein that consists of ECFP, the calmodulin-binding peptide M13

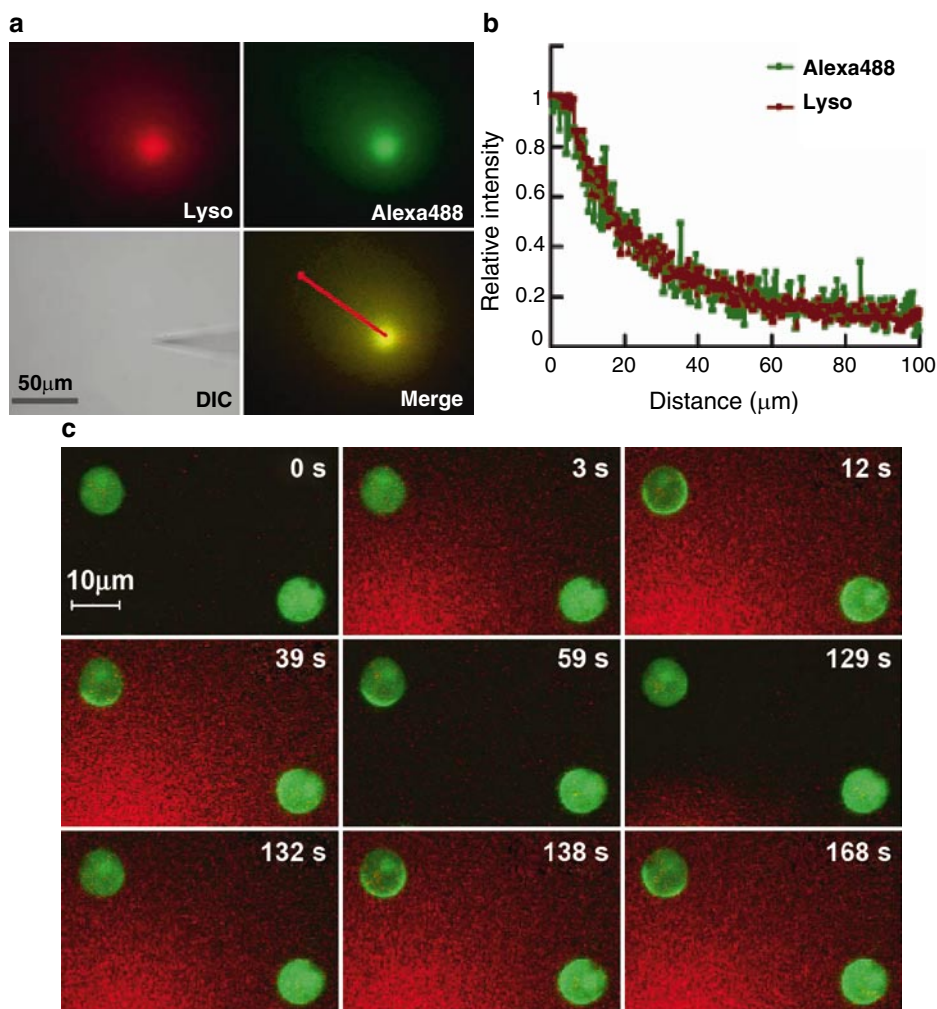


Fig. 1. A stable gradient generated by a micropipette. **(a)** A micropipette filled with a mixture of LysoTracker Red DND-99 (MW 399.025, *red*) and Alexa fluor 488 (MW 643.41, *green*) generated a stable gradient. Fluorescence images of LysoTracker Red DND-99 (*red*) or Alexa fluor 488 (*green*) of the gradient were recorded simultaneously in two channels. Channel one: excitation is 543 nm and emissions from 585 to 615 nm to specifically monitor LysoTracker Red DND-99; Channel two: excitation is 488 nm and emissions from 505 to 530 nm to specifically monitor Alexa fluor 488. **(b)** A comparison of the diffusion concentration profiles for two dyes, LysoTracker Red DND-99 and Alexa fluor 488. Quantitative measurement of the gradient along the line starting from the position of the dispensing micropipette is shown as intensities of LysoTracker Red DND-99 (*red*) and Alexa fluor 488 (*green*) as a function of the distance (μm). Two curves (*red* and *green*) overlap, indicating that difference in their molecular masses and structure had little effect on the profile of the gradient under our experimental condition. **(c)** A time-lapse experiment shows suddenly exposing, withdrawing, and then re-exposing a cAMP gradient (*red*) to two live cells. Two cells expressing PH_{Crac}-GFP (*green*) were suddenly exposed to a cAMP gradient (1 μM) at 0 s for 50 s. The gradient was withdrawn at 50 s and reapplied at 129 s. PH_{Crac}-GFP membrane translocation is visualized in the cells.

and EYFP (14). An increase in Ca²⁺ concentration results in a conformational change in the M13 peptide that decreases the distance between ECFP and EYFP, which, in turn increases the intramolecular FRET signal. Outlined here is a spectrally resolved,

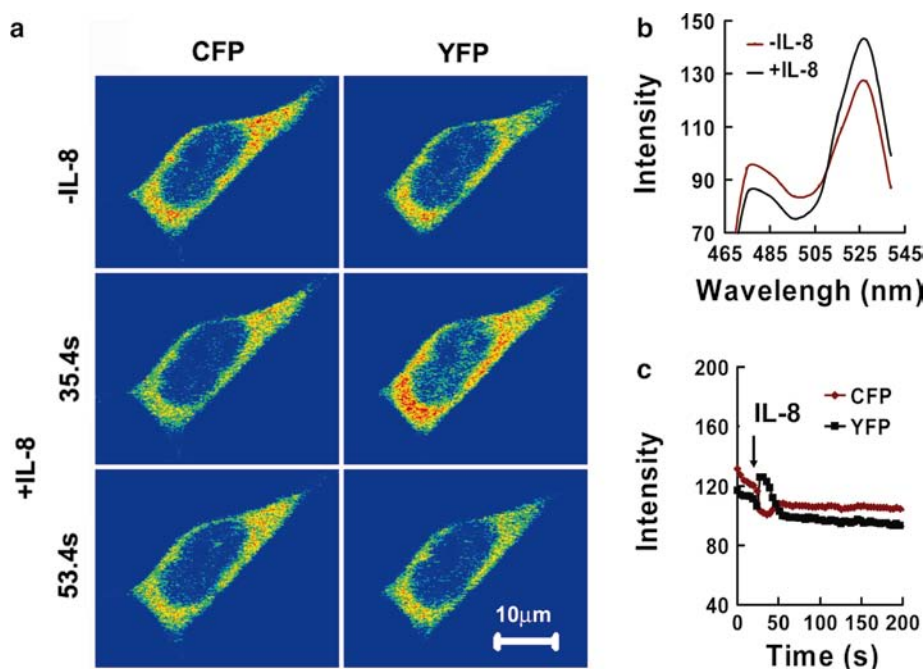


Fig. 2. IL-8 chemokine-induced FRET changes of the fluorescent biosensor for Ca^{2+} , cameleon, in a living cell. (a) Ca^{2+} FRET imaging of a cell expressing cameleon. Stimulation by IL-8 triggers a transient FRET increase of YC6.1, a chimeric protein consisting of CFP, the calmodulin-binding peptide M13 and YFP, indicating an intracellular Ca^{2+} spike. Images of CFP and YFP of cameleon probe in live cells reflecting the Ca^{2+} levels of the basal (-IL8), the stimulated (35.4 s) and poststimulated (53.4 s). IL-8 was added after the third scan. Images were captured at 5-s intervals. (b) Emission spectra of the cell were acquired at an excitation wavelength of 458 nm showing the FRET increase of cameleon with (black line) or without (red line) stimulation by IL-8. A significant increase in the YFP emission signal near 528 nm and a parallel decrease in the CFP emission near 475 nm were observed. (c) Quantitative measurement of temporal intensity changes of CFP and YFP in the cell. The YFP increase and the CFP decrease reflect the IL-8 stimulated intracellular Ca^{2+} spike.

FRET time-lapse imaging method to measure the induced Ca^{2+} response by monitoring intensity changes of ECFP and EYFP in single live HEK293 cells after IL-8 (chemokine) activation of the CXCR1 receptor (Fig. 2).

1. Obtain an emission fingerprint of ECFP and EYFP in live cells. HEK293 cells expressing ECFP or EYFP were excited with a 454-nm laser line, and the spectral emissions in each pixel of the fluorescent images were recorded in eight channels, each with a 10-nm width, from 464 to 544 nm using the Lambda Stack Acquisition mode of LSM 510 META microscope. To obtain the emission fingerprint, we chose a region of interest (ROI) for the images obtained for ECFP or EYFP, and created an intensity plot over the spectral range collected in the Lambda Stack. Each plot was used as ECFP and EYFP fingerprint for the Linear Unmixing function (see Note 4).
2. Obtain spectrally resolved images of cells expressing the FRET pair in Lambda Stack mode using the same conditions as in step 1 before and after IL-8 stimulation (see Note 5).

3. The increase of intracellular Ca^{2+} triggers a FRET increase of cameleon. This change can be monitored as an increase of EYFP (acceptor) signal and a simultaneous decrease of ECFP (donor) signal (**Fig. 2**). Using the spectra of ECFP and EYFP as the references, the time-lapse images of ECFP and EYFP were digitally separated by the Linear Unmixing Function. The intensities of ECFP and EYFP in the ROI in the time-lapse experiment were normalized and expressed as a function of time in response to an IL-8 stimulation (**Fig. 2**, *see Note 6*).

The observed intensity dynamics of ECFP and EYFP in response to IL-8 stimulation showed similar kinetics to IL-8 induced Ca^{2+} response as detected by the Ca^{2+} dye, Fluo4 (**15**). Thus, the spectrally resolved FRET microscopy approach is feasible for visualizing and quantifying fast, reversible protein–protein interactions in single live cells with high temporal and spatial resolution. We have successfully used this method to measure the kinetics of cAR1-mediated G-protein dissociation (activation), association (inactivation), and redissociation (reactivation) in single live cells in response to temporal changes in concentration of the chemoattractant cAMP (**10, 11**).

3.4. Imaging the Dynamics of GPCR and G-Protein Subunits at the Single-Molecule Level by TIRF Microscopy

Using TIR-FM, we have imaged GPCR and G-protein $\beta\gamma$ subunits in the plasma membrane at a single-molecule level in live cells in real time to determine diffusion properties of these proteins. An example of single-molecules imaged at 30 frames per second with an EM-CCD on an IX81 TIR fluorescent microscope is shown in **Fig. 3**. Fluorescence spots of cAR1-YFP were tracked and analyzed from a stack of 100 images with a two-dimensional Gaussian profile method using software described in chapter 29 and two-dimensional trajectories can be calculated by the positional shift in consecutive images (**Fig. 3**). *See* Chapter 29 in this book for further discussion. The following is a detailed procedure for single-molecule imaging of molecules tagged with the YFP fluorophore using TIR-FM.

3.4.1. Preparation of the Cover Glass

1. Use tweezers to put VWR microcover glasses (24×50 mm) into the slots of a Coplin Jar containing Nano Strip. Minimum cleaning time is 30 min.
2. Transfer the cover glasses to another Coplin Jar filled with double distilled water. Exchange the water at least ten times (*see Note 7*).
3. Transfer the cover glasses into another Coplin Jar filled with 200 Proof Ethanol for 10 min.
4. Remove a cover glass at one corner with tweezers and blow filtered argon gas through a pipette over the cover glass to rapidly dry. Alternatively, compressed air from an AccuDuster III can be used, but care must be taken as to not spray propellant

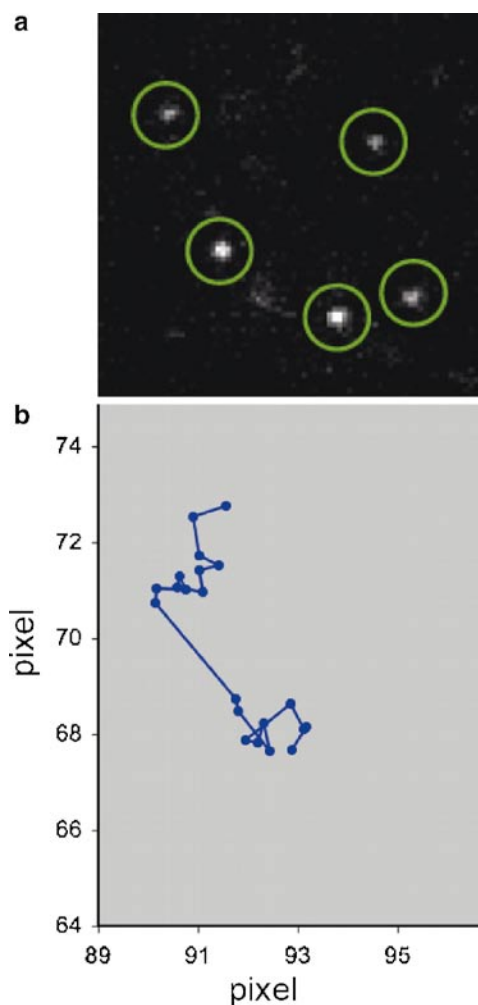


Fig. 3. Single-molecule imaging and analysis of cAR1-YFP. **(a)** A fluorescence image showing five cAR1-YFP molecules. **(b)** The trajectory of an individual cAR1-YFP molecule imaged at 30 frames per second.

onto the cover glass. Place the cover glass onto a monolayer of clean Roll & Grow Plating beads in the Petri dish for storage (see [Note 8](#)).

5. Use the upside to seed the cells for imaging (see chamber preparation later).

3.4.2. Preparation of Labtek Eight-Well Chambers

1. Peel off the cover glass from the bottom of the Labtek chamber.
2. Squeeze equal amounts of parts A and B of Sylgard 164 silicone elastomer onto a flat surface and mix thoroughly.
3. Flip the chamber and put an appropriate amount of the silicon solution into the slot of the chamber using a flat tip or a wide-bore syringe. Do spill over the edge of the slot or leave bubbles in the silicon.

4. Press the cover glass with tweezers to make sure that the cover glass is closely attached to the chamber. After about 30 min, the chamber is ready to seed cells. Up to six chambers can be glued before another batch of silicon would need to be mixed.

3.4.3. Preparation of Cells

1. Apply 0.2 mL of cell suspension to each well of the Lab-Tek II eight-well chamber to achieve a density of $1-3 \times 10^4$ cells/cm². Allow cells to adhere for 10 min; carefully pipette off the buffer to remove unattached cells and replace with the same volume.

3.4.4. Adjustment of the Laser Beam Angle to Achieve TIR

1. Select the 100 $\times$ /1.45 N.A. objective lens, apply immersion oil, and mount the chamber containing cells.
2. Select an appropriate dichroic mirror to reflect the 514-nm excitation light to the specimen. Select an appropriate bandpass filter in the emission filter wheel (550/40, Chroma). In addition to the emission filter, we place a 514-nm notch filter (Semrock) into the emission filter slot of the dichroic mirror cube to block unwanted excitation light from reaching the detector, and we use a 514-nm excitation filter to clean up unwanted wavelengths from the argon gas laser that are not blocked by our acousto-optical tunable filter.
3. Set the light path to the camera and acquire a live image with a 35-ms exposure and focus the sample.
4. Tilt the white light swing arm back and focus the laser beam in the back focal plane of the objective by moving the fiber optic mount holder in the TIR-FM illumination port. When focused, the excitation light exits the lens as a column of light and will project as a tight spot on the ceiling (*see* **Notes 9–11**).
5. Find the critical angle by turning the micrometer on the TIR-FM illumination port CCW. The image intensity will increase and then decrease as the critical angle is approached. We find it best to go beyond the critical angle (the image intensity will be lost) and then turn the micrometer CW while simultaneously adjusting focus on the region of the cell attached to the cover glass.
6. Crop the field of view to minimize the file size and acquire an image stream with a 35-ms exposure time.

4. Notes

1. To ensure an accurate measurement, fluorescence images are usually acquired with a Z-axis resolution at about 1–2 μ m.
2. To stably generate a gradient from a micropipette, the solution of cAMP and a dye solution must be centrifuged at a high speed

for 2 min to remove particulates before loading a micropipette with the solution.

3. Movement of the micropipette from position 1 to position 2 usually takes less than 1 s. To capture changes of cAMP concentration around a cell, we image fluorescent dye using a time-lapse movie with a 1-s interval.
4. The emission fingerprints of ECFP and EYFP are recorded using the identical imaging setting that records FRET sample. The fingerprints are used as references to digitally separate signals of ECFP and EYFP in images of FRET sample by the Linear Unmixing function in LSM 510 META software.
5. Cells expressing ECFP and EYFP were excited with a 458-nm laser line and the spectral emission in each pixel of the images was simultaneously recorded in eight channels, each with a 10-nm width, from 464 to 544 nm. If a larger spectral range is chosen, for example 16 channels (from 464 to 624 nm), two scans are required to cover the wavelengths. Covering a larger range of the wavelengths requires a longer scanning time, which may not be ideal for fast time-lapse imaging.
6. Using this method, FRET changes can be easily seen by emission spectra, which shows an increase (or decrease) of ECFP signal and a simultaneous decrease (or increase) of the EYFP signal. In addition, intensity changes of ECFP and EYFP in the digitally separated images also indicate FRET changes.
7. Single-molecule imaging requires an ultraclean cover glass. Nano Strip (Cyantek Corporation) is a powerful cleaning solution. Be extra cautious about safety.
8. Roll & Grow plating beads are cleaned using the same procedure for cleaning the cover glass.
9. The column of light can be readily observed through a well filled with fluorophore.
10. It is important to have the lens in a position where the specimen is focused; if the lens height is changed significantly, the excitation laser line will no longer retain focus.
11. Removing the specimen can help in determining the best focus while referring to the spot projected on the ceiling, and may be necessary in cases where the cover glass has been densely seeded with cells as this will scatter the excitation beam.

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Chapter 26

Imaging Actin Cytoskeleton Dynamics in *Dictyostelium* Chemotaxis

Günther Gerisch

Summary

This chapter will focus on responses that the chemoattractant cyclic AMP elicits in the motility system of *Dictyostelium*. These cells can be permanently transfected to express cytoskeleton-associated proteins tagged with fluorescent proteins. Multiple proteins that are distinguishable by the excitation and emission spectra of their tags can be simultaneously expressed. This makes it possible to relate the spatial and temporal patterns of their chemoattractant-induced translocation to each other in one cell by a single recording. Since actin polymerization in live cells progresses with velocities of about 3 $\mu\text{m/s}$, high image frequencies and short acquisition times in the millisecond range are required. Techniques of total internal reflection fluorescence (TIRF) and spinning-disc confocal microscopy provide appropriate temporal and spatial resolution for the analysis of actin dynamics.

Key words: Actin polymerization, Arp2/3 complex, Coronin, G-protein-mediated signal transduction, Myosin, Spinning-disc confocal microscopy, Total internal reflection fluorescence microscopy

1. Introduction

1.1. Overview of Rapid Actin Responses in Chemotaxis

The chemotactic response of *Dictyostelium* cells, and certainly also of other amoeboid cells such as neutrophils, is superimposed on the spontaneous dynamics of the cytoskeleton, in particular of the actin system in the cell cortex. This system consists basically of a network of bundled actin filaments, which are continuously turned over by polymerization and depolymerization. In *Dictyostelium*, the polymerization rates of actin filaments constituting the network are in the order of 3 $\mu\text{m/s}$, corresponding to the

addition of more than 1,000 actin monomers to one filament per second (1).

Locally integrated into the network are structures associated with specific proteins that modify the arrangement of actin filaments. At leading edges, and also at phagocytic or macropinocytic cups and at sites of clathrin-dependent endocytosis (2), a dense fabric of filamentous actin is built in association with the Arp2/3 complex (1, 3). Filopodia are extensively formed and retracted in chemotaxing cells (1). They are characterized by a set of proteins that bundle actin filaments in parallel. At the tip of filopodia, a complex of proteins is assembled including VASP and myosin-VII, which are required for the formation of these finger-like protrusions (4, 5).

In order to cope with the high rate of actin reorganization in stimulated as well as unstimulated cells, the visualization of actin structures requires acquisition times of 100 ms or less. Accordingly, for the investigation of actin dynamics frame rates of 10 per second or, for a detailed analysis, even higher rates are needed. Here techniques appropriate to visualize dynamic actin structures in chemotaxing cells will be reviewed, with an emphasis on dual-wavelength imaging using either total internal reflection fluorescence (TIRF) or spinning-disc confocal microscopy. These techniques are based on the simultaneous expression of green and red fluorescent proteins within the same cell. The design of vectors and the optimal tagging of proteins to be expressed in *Dictyostelium* cells has recently been covered by Müller-Taubenberger (6).

1.2. Cell Strains Expressing Fluorescent Proteins

Most of the vectors and transformed cells needed for the experiments described are available from the *Dictyostelium* Stock Center (<http://dictybase.org>). GFP is the enhanced S65T version, mRFP is mRFP_{Mars} optimized for expression in *Dictyostelium discoideum* (7).

1.2.1. Fluorescent Actin Labels

GFP-Actin

The actin 8 gene encodes the predominant actin species in *Dictyostelium* cells. With GFP attached to its N-terminus, this actin polymerizes together with untagged actin. If the ratio of tagged to native actin is below 10%, no major reduction in the rate of polymerization is observed in vitro and cells expressing the fluorescent actin are not obviously impaired (8).

Since about half of the actin exists in an unpolymerized state in *Dictyostelium* as in other cells, the direct labeling of actin is unavoidably deteriorated by a high cytoplasmic background. Therefore, the direct labeling of actin is only advisable if the purpose of the experiment does demand it. Measuring the turnover rate of filamentous actin structures by FRAP is one of these purposes.

Fluorescent LimEΔ Constructs	An optimal label for filamentous actin structures should bind selectively and with high affinity to filamentous actin. On the other hand, the dissociation rate should be high enough for the label to accommodate to the dynamics of actin structures in vivo. For <i>Dictyostelium</i> cells, we found fluorescent constructs of the LimE protein most appropriate (9). The LimE protein of <i>Dictyostelium</i> is an actin-binding protein comprising three domains: a Lim domain, a glycine-rich region, and a coiled-coil tail. The two first domains are essential for actin binding, whereas deletion of the tail even improves the quality of labeling. The construct lacking the C-terminal coiled-coil is designated as LimEΔ. It has a molecular mass of 15 kDa and can be tagged at its N- or C-terminus with GFP. For dual-wavelength imaging in concert with GFP-tagged proteins, mRFP-LimEΔ or Cherry-LimEΔ can be used (see Note 1).
1.2.2. Proteins Associated with the Leading Edge in Chemotaxing Cells	The three proteins discussed in this section label different sites at a leading edge and respond differently to the protrusion and retraction of leading edges during the reorientation of cells in gradients of chemoattractant. All three proteins associate also with phagocytic cups and sites of clathrin-associated endocytosis (2).
Myosin-IB at the Cutting Edge of Lamellipodia	The single-headed myosin-IB can be tagged at its N-terminus with GFP (Margaret Titus, unpublished result). GFP-myosin-IB localizes transiently to the very border of leading edges which are protruded in response to chemoattractant.
Subunits of the Arp2/3 Complex	Arp2/3 labels are concentrated in regions of the cell cortex that are densely packed with filamentous actin. Among the seven members of this complex two subunits, Arp3 and ARPC1, have been successfully tagged with fluorescent proteins at their N-terminus without abolishing localization to actin structures in <i>Dictyostelium</i> cells (2). These subunits differ from each other in their association with the Arp2/3 complex: whereas Arp3 is tightly bound to the complex, ARPC1 is the only subunit that readily exchanges between the bound and free state (10).
Coronin, an Indicator of Actin Depolymerization	Coronins are WD40-repeat proteins that form 7-bladed propellers (11). <i>Dictyostelium</i> coronin is strongly enriched at leading edges (12). Detailed analyses of its localization in phagocytosis (13), cell spreading (2), and chemotaxis indicated that this coronin is recruited to actin structures that are in the process of disassembly. The coronin can be tagged at its N- or C-terminus without impairing its localization.
1.2.3. Labeling Filopodia	In chemotaxing cells of <i>Dictyostelium</i> , the filopodia are capable of rapidly extending and retracting, of bending and attaching with their tips to a substrate surface (1). Because of the small diameter

of filopodia in the 100–200 nm range and the presence of actin filaments along their entire length of several micrometers (14), labeling of filamentous actin is the method of choice to study filopod dynamics. LimEΔ-GFP or mRFP-LimEΔ proved to be excellent markers for filopodia (1). The tip of filopodia is selectively labeled with GFP-myosin-VII (5), and this label can be combined with mRFP-LimEΔ (see Note 2).

1.3. Practical Considerations for Imaging Actin Dynamics

1.3.1. Expression Levels

Since actin structures are assembled from G-actin present in the cytoplasm, and most of the proteins associated with these structures are also recruited from the cytosolic pool, a cytoplasmic background of these proteins will always be present. The rule is to keep the expression of any fluorescent protein not higher than necessary for the imaging of its structure-bound fraction. Strong expression would not only cause the risk of overexpression artifacts (see Note 3) but would also increase the cytoplasmic background. For instance, incorporation of the GFP-tagged Arp3 subunit into the functional Arp2/3 complex will be limited by the endogenous concentration of the other six members of the complex.

Small proteins may pass the nuclear pores. For instance, mRFP-LimEΔ slightly accumulates in the nucleus relative to the cytoplasm. It should also be taken into account that a tagged protein may localize appropriately, but lacks function and even interferes with the activity of its untagged endogenous counterpart. An example is C-terminally tagged actin, which incorporates into actin filaments but blocks the movement of myosin along these filaments (15).

For the integrating vector constructs we are using to express fluorescent proteins under control of the actin 15 promoter, there are two ways of modulating the expression level. First, to select an appropriate clone after transformation of the cells (for details see (6)). These vectors integrate randomly into the genome, and the level of expression depends on the site of integration. Second, even in a clonal population the expression varies from cell to cell. If green and red fluorescent proteins are expressed simultaneously, their levels fluctuate independently. Therefore, the experimentalist has to choose cells within a population that exhibit the optimal levels of expression (see Note 4).

1.3.2. Background Fluorescence

Intrinsic fluorescence of the cytoplasm at the wavelengths used for green or red fluorescent proteins is low enough for being neglected in the imaging of actin structures, provided the fluorescent protein of interest is sufficiently expressed. Endosomes filled with nutrient medium are the major source of intrinsic fluorescence. Their fluorescence is of no concern in TIRF images, since the endosomes are located to the interior of the cells out of the range of the evanescent field. In confocal images the contents

of the endosomes will be recognized in cells cultivated axenically in HL5 or similar media containing yeast extract. If this is a problem, low-fluorescent media (16) may be used, though the cells grow then more slowly or saturate at lower density. Alternatively, the cells may be cultivated on bacteria such as *E. coli* B/2.

1.3.3. Introducing a Reference for the Cell Border

In imaging the 3-dimensional organization of the actin system by confocal microscopy, the location of a structure relative to the plasma membrane may be of interest. Therefore, if images are reconstructed in z-direction from stacks of confocal sections, a reference to exactly locate the cell-to-substrate interface is advisable.

Labeling the Plasma Membrane

For labeling the plasma membrane, a series of styryl dyes with different spectral properties is available (17). FM4-64, FM2-10, and FM1-43 have been tested for the use with *Dictyostelium*. For the combination with GFP-tagged proteins, FM4-64 offers the advantage of being excitable at the same wavelength (488 or 491 nm), and easily separated from GFP by its emission peak above 700 nm (14). All these styryl dyes label first the plasma membrane and then redistribute within a few minutes to membranes of the contractile vacuole system and of endosomes. Therefore, they should be added to the cells immediately before imaging of the plasma membrane is attempted. Since cells tend to round up as the dyes are integrated into the lipid layer of the membrane, concentrations should be kept at the lowest limit.

Marking the Cell-to-Substrate Interspace

Since fluorescent dextran of molecular mass 3,000 or higher does not penetrate the plasma membrane, it can be used to label the space between the substrate surface and the bottom of an attached cell, thus providing a reference for the extension of an actin structure into the cytoplasmic space (18, 19). Cells capable of growing axenically, gradually take up the dextran by macropinocytosis until after an hour the contents of vesicles in the entire endosomal pathway are labeled.

1.4. Imaging Techniques

1.4.1. Fast Recording of Actin Dynamics by TIRF or Spinning-Disc Confocal Microscopy

Imaging of actin dynamics in live cells requires optical sectioning, image acquisition in the millisecond range, and lowest possible light exposure to minimize cytotoxicity and photobleaching (see Note 5). These requirements are met by TIRF and by spinning-disc microscopy. In both techniques, the entire region of interest in a microscopic field is projected onto a camera chip. In TIRF microscopy, a shallow layer close to a reflecting surface is illuminated by an evanescent field. Spinning-disc microscopes, other than line-scan microscopes, make it possible to illuminate a microscopic field almost synchronously in the confocal mode. For high-frequency recording, a frame-transfer camera of high sensitivity and resolution will be needed. Because appropriate equipment is available from various suppliers, and progress in the

optical setup, in camera sensitivity and resolution, and in image-processing software is fast, it seems useless to suggest one particular equipment currently on the market. A schematic setup is shown in **Fig. 1**, and the equipment used in our own studies has been specified in detail (2, 19). In the following sections, advantages and drawbacks of each of the fast imaging techniques will be outlined.

TIRF Microscopy

The evanescent field used for fluorescence excitation in TIRF microscopes declines exponentially with increasing distance from the reflecting surface. In practice, fluorescence will be recorded from a layer extending about 100 nm from the plasma membrane of an attached cell into its interior, this means with minimal background contributed by the bulk of the cytoplasm. The cortical actin network of *Dictyostelium* cells is located within the layer illuminated by the evanescent wave. Therefore, TIRF microscopy visualizes the actin network structures with a brilliance that is unmatched by any other technique.

Disadvantages reside in the ambiguous relation of fluorescence intensity to the amount of fluorescent protein present.

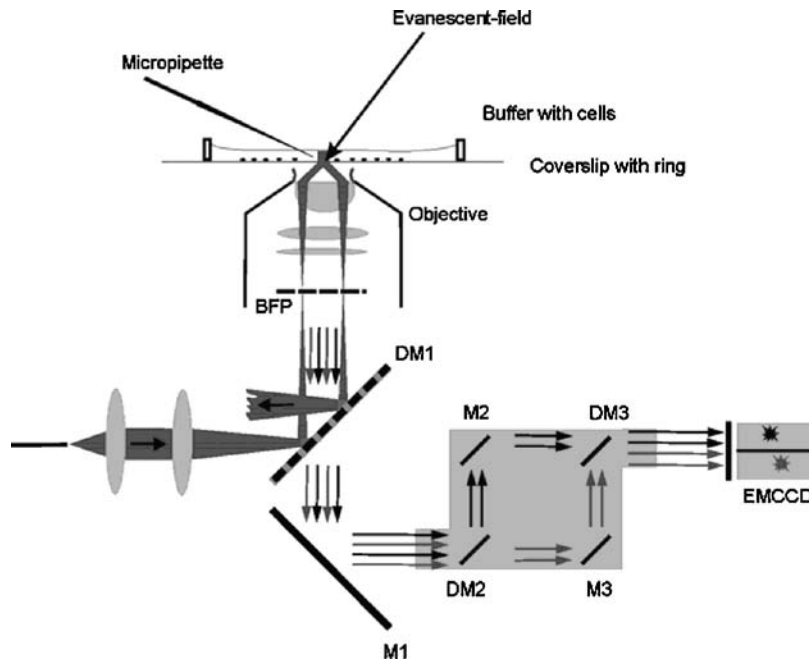


Fig. 1. Principle of dual-emission TIRF imaging of chemotaxing cells. Cells moving on a coverslip within a plexiglass ring are stimulated through a micropipette with cAMP. An evanescent field is generated by illumination with 488-nm or 491-nm laser light (BFP back focal plane). A dichroic mirror *DM1* separates excitation from emission wavelengths. *DM2* is a shortpass dichroic, *DM3* a longpass, both separating GFP and mRFP emissions. Images produced by GFP emission (gray arrows) are separated from images produced by mRFP emission (black arrows) on two halves of the camera chip. Filters that increase selectivity are not shown. Courtesy Chris Gell, MPI of Molecular Cell Biology and Genetics, Dresden.

Since the fluorescence excitation in an evanescent field depends on the distance from the reflecting surface, only the intensities of proteins at the same distance can be directly compared, for instance the intensities at regions of the plasma membrane that adhere to the substrate. For chemotaxing cells one has to keep in view that the very front of a cell is not always attached to the substrate and therefore may not be seen in TIRF images.

Since the penetration of the evanescent field into the cell depends on the wavelength of excitation, structures remote from the membrane may be seen at 568-nm but not 488-nm excitation. This ambiguity is avoided if two fluorophores are excited at the same wavelength and discriminated by their emission spectra. mRFP is weakly but often sufficiently excited at 488 nm for this wavelength to be used for both mRFP and GFP. For dual-wavelength TIRF imaging it is therefore recommended to combine GFP with mRFP rather than Cherry.

Spinning-Disc Microscopy

Confocal imaging using spinning-disc equipment is the technique of choice for recording structures apart from the substrate surface and for evaluating protein distributions in z-direction (*see* [Note 6](#)). However, taking into account that a single actin filament has a diameter of 8 nm and even with high-aperture objectives the point-spread function in z-direction has a width at half-maximum of $\geq 0.7 \mu\text{m}$, it is obvious that delicate actin structures are less precisely portrayed than by TIRF.

An overview of all actin-based activities in a cell is most conveniently obtained by maximum projection of z-stacks comprising the entire cell. Confocal sections are superimposed on each other, and in each row of pixels the highest fluorescence intensity is determined (<http://rsbweb.nih.gov/ij/→open> image stack→stacks→3D Project). **Figure 2** shows that by maximum projection the various actin structures present in different regions from bottom to top of a chemotaxing cell are simultaneously viewed.

1.4.2. Choice of Simultaneous or Consecutive Image Acquisition in Dual-Wavelength Imaging

In both TIRF and spinning-disc microscopy, there are two ways of acquiring multiple fluorescence channels in order to relate the localizations of different proteins to each other: the images can be acquired either consecutively or simultaneously. Requirements and shortcomings of these modes of imaging are outlined as follows.

Consecutive Image Acquisition

If images of two fluorophores are recorded one after the other, the channels may be separated by switching the lasers for excitation and/or by changing the filters at the side of emission. Changing excitation wavelengths by an optoacoustic switch is fast, changing emission by a filter wheel may cause a delay of 100 ms. Therefore the channels should be separated by the excitation wavelength only, for instance 488 nm for GFP and 568 nm for Cherry or mRFP. Cherry is preferable since it is negligibly excited at 488 nm. Since mRFP is weakly excited at 488 nm, its fluorescence

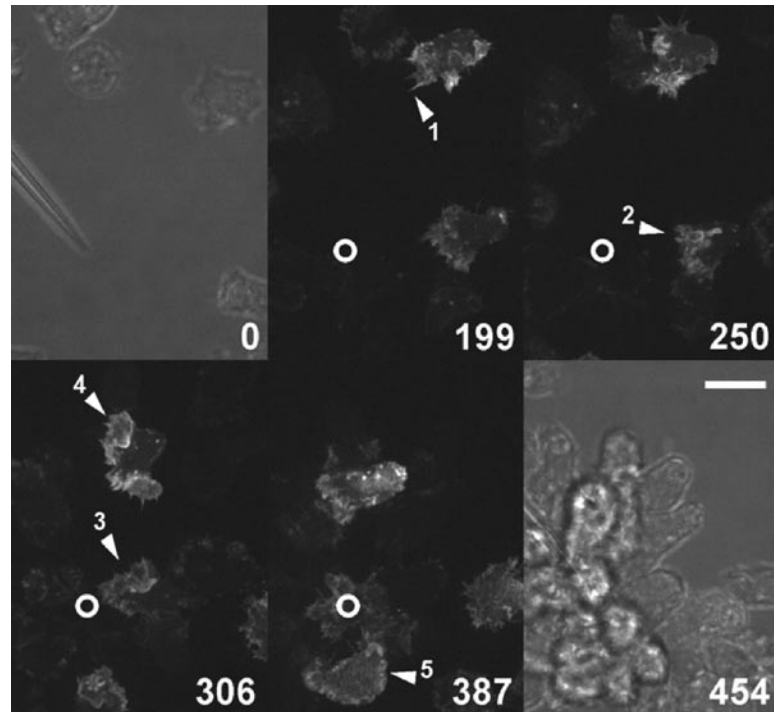


Fig. 2. Filamentous actin structures in cells stimulated by attractant. Cells of *D. discoideum* migrate toward a micro-pipette filled with cAMP (first bright-field image) and accumulate there within 8 min (last image). Actin structures are labeled with LimEΔ-GFP and viewed throughout the entire cell bodies by maximum projection: 1, filopodia; 2 and 3, macropinocytic cups showing membrane internalization at the front and other sites of the cell surface; 4 and 5, actin waves propagating on the substrate-attached cell surface. Time is indicated in seconds, tip position of the pipette by circles. Bar, 10 μ m. In cooperation with Ulrike Engel at the Nikon Imaging Center, University of Heidelberg.

will significantly contribute to emission in the GFP channel. To correct for this contribution, the factor of crossexcitation is determined by exciting at 568 and 488 nm a specimen labeled only with mRFP. By applying this factor, the image obtained at 568-nm excitation is subtracted from the image obtained at 488 nm. The camera setting should be the same in control and experimental recordings.

Even with an optoacoustic switch there will be a delay between two images that depends on the acquisition time required for each channel. For fast processes in the cytoskeleton this delay will be relevant. For instance, movements driven by motor proteins are in the order of several micrometers per second.

Simultaneous Image Acquisition

The emissions of two fluorophores are separated by a dichroic in order to record two fluorescent proteins synchronously, as required for the exact superposition of proteins participating in highly dynamic processes. If the two channels are not precisely in the same focus, a lens should be inserted into one channel.

Each channel is then projected onto one-half of the camera chip, which means that image sizes are reduced to half of the chip.

For the correlation of protein locations in two simultaneously recorded images, the two channels need to be precisely superimposed on each other. To determine the coordinates for adjustment in the x, y-plane, a control image is recorded of red-green fluorescent beads dispersed in the microscopic field, and the images obtained in the two channels are merged. If aberrations are nonlinear, the program <http://www.kalaimoscope.com> (20) will provide an optimal fit.

1.5. Recording Actin Dynamics in Response to Chemoattractant

In order to apply the imaging techniques outlined earlier to changes in the actin cytoskeleton that are induced by cAMP, the cells have to be cultivated and starved in a way that they acquire full responsiveness to the chemoattractant. For stimulation, cells should be exposed to fast changes in gradient direction or attractant concentration while they are continuously recorded under the microscope. In the following chapters two techniques will be described, one of exposing cells to spatial gradients of attractant, the other to temporal changes in the concentration of attractant.

2. Materials

2.1. Cell Culture

1. Nutrient medium (1 L): 7.15 g yeast extract (Oxoid), 14.3 g bacteriological peptone L37 (Oxoid) or neutralized peptone (Oxoid), 18.0 g maltose (Merck), 0.486 g KH_2PO_4 , 0.616 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, adjusted to pH 6.7, and autoclaved at 120°C (see Note 7).
2. PB, 17 mM phosphate buffer, pH 6.0 (1 L): 2.0 g KH_2PO_4 , 0.356 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, autoclaved at 120°C. This “phosphate buffer” is generally used for imaging and starving of *D. discoideum* cells.
3. Blastidicine S hydrochloride (Fluka), 10 mg/mL stock in phosphate buffer, store aliquots at –20°C.
4. Geneticin (Sigma-Aldrich), 20 mg/mL stock in phosphate buffer, store aliquots at –20°C.
5. Hygromycin B (Calbiochem), 33 mg/mL stock in phosphate buffer, store aliquots at –20°C.
6. Petri dishes, 10 and 3.5 cm in diameter (Falcon).

2.2. Fluorescence Microscopy

1. Inverted microscope with EMCCD camera, equipped for TIRF or spinning-disc microscopy.
2. Optical filters, e.g., from Chroma Technology Corp. (<http://www.chroma.com>).

3. Alexa 680 (Invitrogen), to be mixed with cAMP to 50 µg/mL final concentration in phosphate buffer.
4. Green/red fluorescent beads, Tetra Speck bead slide (Invitrogen).
5. Fluorescent dextran: Texas red dextran 3000, 2 mg/mL, or Alexa-Fluor dextran 10,000, 0.5 mg/mL final concentration in phosphate buffer (Invitrogen). Stock solutions stored at -20°C .
6. FM4-64 (Invitrogen). A stock solution of 1 mg/mL in DMSO is kept at -20°C . Immediately before use, 10 µL is slowly diluted with 20 mL phosphate buffer. This solution is mixed 1 to 1 with phosphate buffer containing the cells (final concentration: 250 ng/mL). The dilution is not stable.

2.3. Stimulation with Chemoattractant

1. Femtotip glass microcapillaries (Eppendorf).
2. Microloader tips (Eppendorf) to fill the Femtotips from behind.
3. Adenosine 3', 5'-cyclic monophosphate sodium salt monohydrate (cAMP) (Sigma-Aldrich), 1 mM in phosphate buffer. Store 100 µL aliquots at -20°C . Dilute before use to 10^{-4} mM with phosphate buffer.
4. Glass coverslips # 1.5, 50 × 50 mm.
5. Plexiglass rings, 40 mm diameter, height 4 mm, wall 5 mm.
6. Paraffin, 52–54°C (VWR) to seal the plexiglass rings onto the coverslips.
7. Micromanipulator, e.g., Eppendorf Micromanipulator 5171 with micropipette holder.
8. Flow chamber, channel size 17 mm length, 3.8 mm width, 0.4 mm height. Ibidi µ-Slide VI glass bottom, number 80608, with Luer connection to flow-through kit (<http://www.ibidi.de>).
9. Peristalsis-free pump, PHD 2000 Advanced Syringe Pump (Harvard Apparatus). For the stimulation of development with cAMP pulses, 10-mL syringes are connected with a hypodermic needle to 0.75 × 1.22 mm polyethylene tubing (LABOKRON 46470).

3. Methods

3.1. Cell Growth and Development to Aggregation Competence

Laboratory strains like AX2 or AX3 efficiently take up liquid by macropinocytosis, and grow in nutrient medium with a generation time of 8–9 h at 21–23°C. Their parent wild isolate NC4 depends on the uptake of bacteria by phagocytosis, and grows on *E.coli*

B/r or B/2 with a generation time of 3–4 h. To induce development to the aggregation-competent stage, the cells are harvested during exponential growth, washed, and starved in non-nutrient buffer (see [Note 8](#)). Since the cells stimulate their own development by the periodic generation of cAMP pulses, the time they need for development will strongly depend on their density.

3.1.1. Simplified Procedure

1. Day 1. Spores or cells of the *D. discoideum* strain AX2, or mutants derived from this strain, are transferred to 10 mL nutrient medium in a 10-cm-diameter petri dish and incubated at 23°C.
2. Day 2. To apply selection pressure on the expression of fluorescent proteins appropriate antibiotics are added.
3. Day 3 or 4. The cells are harvested before monolayers become confluent. For one chemotaxis experiment at least two petri dishes are needed.
4. The cells are gently suspended and centrifuged in a plastic tube for 4 min in a swing-out rotor at $200 \times g$ and 4°C. The pelleted cells are resuspended and washed thrice on the centrifuge in 10 mL cold PB.
5. The pelleted cells are brought up in phosphate buffer to a density of 3×10^6 cells per mL and dispensed in 5-mL aliquots into petri dishes of 3.5-cm diameter.
6. Chemotactic responsiveness is attained at 23°C after 6–8 h of starvation, when the cells just start to elongate and assemble into streams.

3.1.2. Standardized Procedure

Cells are cultivated in shaken suspension in nutrient medium and harvested during exponential growth (cell density not higher than 5×10^6 /mL). The cells are washed three times in PB, adjusted in the buffer to 1×10^7 /mL, and 30 mL of the suspension are shaken in 100-mL Erlenmeyer flasks. In strain AX3 the development needs to be stimulated by cAMP pulses, and in strain AX2 the development is enhanced by pulsatile stimulation. For this, cAMP is dropped into the cell suspension every 6 min to 200 nM final concentration at each pulse (for instance, 16 μ L droplets of 4×10^{-4} M cAMP) applied through a pump, e.g., the peristaltic-free pump listed up under **Subheading 2.3, item 9** or a medical perfusor pump.

3.2. Actin Dynamics in Changing Gradients of Attractant

3.2.1. Stimulation Through a Micropipette (see [Note 9](#))

1. 50 μ L of the starved cells is dispensed in 3 mL phosphate buffer and transferred onto a 50 $\times$ 50 mm cover slip with a plexiglass ring of 40 mm inner diameter and 4 mm height. Cells are allowed to settle for 15 min.
2. An aliquot of the 1 mM cAMP stock solution is thawed and diluted to 10^{-4} M with phosphate buffer.

3. An Eppendorf Femtotip is filled free of air bubbles with 10^{-4} M cAMP using the Eppendorf Microloader Tips. The pipette should be completely filled to prevent streaming of the solution by capillary forces. The micropipette is fixed on a micromanipulator at a vertical angle of about 30° to the microscope stage.
4. Some manual skill is needed to place the pipette tip into the microscope field slightly above the cells. To prevent the tip from breaking off, the pipette should never touch the glass bottom. First, the tip region is immersed into the phosphate buffer above the cells and, by viewing from the side, is brought into the light beam of the microscope. With the focus above the cell layer, the pipette is moved laterally until its shadow is seen under the microscope. Then the pipette is stepwise moved back and downward. When the tip is in the microscope field several micrometers above the cells, recording can commence.

3.2.2. Image Analysis of the Gain and Loss of Actin Structures

To analyze actin dynamics, time series are recorded and fluorescence intensities in images are sequentially compared by superposition or subtraction. For qualitative purposes, each image in the series is superimposed on the previous or an earlier image. If the later image of each pair is colored in red and the earlier one in green by RGB processing, the gain of structures will appear in red, the loss in green, and invariant structures in yellow.

For quantitative comparison, images encoded in a linear gray scale at least in the 8-bit mode are subtracted from each other. To evaluate the gain of elements in an actin structure, image n is subtracted from image $n + x$. To evaluate the loss, image $n + x$ is subtracted from image n . To measure the progression of filamentous actin structures in the cortical network of *Dictyostelium* cells, this technique is applied to TIRF image series of cells labeled with LimEA-GFP (1).

3.3. Response to Temporal Programs of Attractant Concentration

D. discoideum cells respond to an upshift of cAMP with a transient increase of filamentous actin in their cortical region and with the recruitment of myosin-IB, the Arp2/3 complex and coronin to this layer (21). These responses are typical of the front of a migrating cell. They can be separated in time from the recruitment of myosin-II, typically accumulating at the retracting tail of a cell: cortical actin reaches a peak at 4 s after the onset of stimulation, myosin-II after about 20 s. In order to record the temporal sequence of responses to cAMP, cells are stimulated in a flow chamber that allows up and down regulation of cAMP concentrations, essentially in the absence of a spatial gradient.

3.3.1. Stimulation of Cells in a Flow Chamber

The flow chamber is fixed on the microscope stage, and the flow produced either by elevating a reservoir beyond the level of the chamber or by a peristalsis-free pump placed downstream of the chamber. The tube connecting the reservoir with the chamber

should be as short as possible to reduce gliding concentration changes by laminar flow in the tube. Since *D. discoideum* cells respond to shear stress similar as to chemoattractant (22), the flow rate should be limited and kept constant. Orientation of cells against the flow direction should be ruled out in controls without attractant. For timing of the temporal changes of attractant at the position of the cells, cAMP is mixed with Alexa 680, 50 µg/mL final concentration (23) or another fluorescent dye of similar molecular mass.

3.3.2. Evaluating the Recruitment of Actin and Associated Proteins to the Cell Cortex

An appropriate read-out of the responses to changing attractant concentrations is the accumulation of polymerized actin and associated proteins in the cortex relative to the interior of the cells (21). This translocation can be quantified by applying an active-contour algorithm based on a flexible chain surrounding the perimeter of a cell (24). The chain is subdivided into 100 equidistant nodes. A second, shorter chain is layered 1-µm deep into the cell, and the area between the chains is defined as the cortical region (Quim P package for the Image J software (<http://rsb.info.nih.gov/ij/>)). The area circumscribed by the inner chain represents the interior of the cell body, and the fluorescence intensities in this area are averaged (see Note 10). One way to quantify fluorescence intensities in the cortex is to draw 100 radial lines from the nodes of the outer chain to the inner chain, and to determine the highest intensity along each line. For calculating the mean intensity in the cortex, the values along each line are averaged. For evaluating polar patterns of protein distributions, these values are plotted in the form of polar coordinates (22).

4. Notes

1. Another construct that binds selectively to filamentous actin is the calponin-like actin-binding domain of ABP120, the *Dictyostelium* ortholog of filamin (25). With GFP added to the N-terminus of this domain, the cytoplasmic background has been somewhat higher in our hands than with LimEΔ-GFP. The domain from ABP120 has the benefit of being known to work also in mammalian cells.
2. An alternative label for the tip of filopodia is GFP-VASP (26).
3. Any protein that binds exclusively or preferentially to polymerized actin should, by mass action law, shift the equilibrium from monomeric to polymeric actin. To avoid artifacts due to this effect, either full-length or truncated LimE constructs may be expressed in LimE-null cells (1).

4. The actin 15 promoter enables proteins to be expressed in the growth phase as well as the aggregation-competent stage of starved cells. If one wants a protein not to be present during growth but expressed in aggregating cells, the strictly regulated contact-site A promoter can be used to drive transcription (27).
5. Cells of *D. discoideum* tend to contract when blue or green light is turned on, apparently by signal transduction from photoreceptors to the actin system (28). Therefore, light-induced changes in actin structure and dynamics should be seriously taken into account by avoiding exposure to the laser light prior to imaging and using the first image as a reference for potential changes in the subsequent ones. In myosin-II-null mutants the contraction is reduced, but not necessarily other alterations in the actin system.
6. For deconvolution, a distance of 200 nm between the optical sections would be optimal. However, for the 3-dimensional reconstruction of a *Dictyostelium* cell, about 60 sections would be needed (for a cell of 10 μm height, including five sections each below and above the cell). Double that number will be required for dual-wavelength imaging. Since chemotaxing cells are moving during acquisition of an image stack, their shape will appear distorted in z-direction. Therefore, one has to compromise about the number of optical sections. Much information on the cortical actin system is already obtained by alternating between two planes, one through the cortex close to the substrate-attached cell surface, the other about 2–4 μm above through the leading edge and the middle of the cell body.
7. Batches of yeast extract and peptone are of varying quality. Each combination of batches should be tested. If found appropriate for growth and development, a sufficiently large quantity of the same two batches should be ordered.
8. *Dictyostelium* cells are obligatory aerobes that must be continuously supplied with oxygen; otherwise the cells bleb, round up, and die within a couple of minutes. Therefore, an inverted microscope is recommended for imaging through a glass coverslip on which the cells are migrating in a fluid layer of about 3 mm height exposed to air, or are covered by an agarose layer. The specimen should be exposed to air or closed on top with a teflon or other membrane permeable for oxygen.
9. Stimulation through a micropipette is a convenient technique that provides steep gradients and allows rapid changes in their direction. For studying cells exposed to two gradients of varying directions, two micropipettes fixed to separate micromanipulators can be moved independently (29).

However, the gradients are not well defined and cells cannot be compressed under a layer of agarose for improvement of the images (30). Defined spatial gradients can be produced in microfluidic chambers (31). Sandwiching between parallel glass and agarose surfaces simplifies the geometry of cells and improves the localization of fluorescent proteins. An agarose overlay can be applied in combination with the uncaging technique that generates cyclic AMP from an inactive precursor by illumination at 405 nm (32). In this case the precursor may be soaked into the agarose before starting the experiment.

10. The interior of the cell should not be taken as a uniform cytoplasmic space. It is filled with compartments separated by membranes: endosomes, the voluminous ER, mitochondria, and the nucleus.

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Chapter 27

Analysis of Actin Assembly by In Vitro TIRF Microscopy

Dennis Breitsprecher, Antje K. Kieseewetter, Joern Linkner, and Jan Faix

Summary

Since directed movement toward an extracellular chemoattractant requires rapid and continuous reorganization of the actin cytoskeleton to form complex structures such as a protruding lamellipodium, it is of great interest to analyze and understand the individual contribution of proteins specifically involved in this process. Over the last decade, enormous progress has been made toward understanding the versatile molecular mechanisms underlying actin-based cell motility and the regulation of site-specific F-actin assembly and disassembly. In spite of this wealth of knowledge and due to the constant discovery of novel regulatory factors, many questions remain to be answered. In this chapter, we describe a powerful method that allows to study the effects of actin-binding proteins on the assembly of single filaments by in vitro total internal reflection fluorescence (TIRF) microscopy using purified proteins and fluorescently labeled actin.

Key words: Actin purification and assembly, Actin-binding proteins, *Dictyostelium*, NEM-myosin II, TIRF microscopy

1. Introduction

Nonmuscle cells contain a large pool of globular monomeric actin, which upon reception of appropriate stimuli and relay by Rho GTPase signaling can reversibly polymerize into filamentous actin and thus trigger numerous cellular activities including the establishment of cell morphology and cell motility. The migration of cells is exceptionally complex and the result of the spatial and temporal coordination of four distinct processes: extension of protrusions such as lamellipodia or pseudopodia, stabilization of these structures by attachment to the substratum, translocation of the cell body, and detachment of the rear part of the cell upon disassembly of cell adhesions at this site. The assembly

and disassembly of actin filaments is accomplished by a plethora of proteins associated with the actin cytoskeleton to effect actin filament nucleation, elongation, branching, capping, and severing as well as actin monomer sequestration and bundling or crosslinking of actin filaments. Elucidating the biophysical and biochemical properties of proteins regulating actin polymerization is therefore important to understand fundamental aspects of cell motility or chemotaxis.

Numerous biochemical in vitro assays for studying actin polymerization have been established in the past, and many of them are still valuable tools for either determining the conditions required for shifting the G- to F-actin equilibrium or measuring how a given protein interacts with actin. Among these are for example the rather simple low- and high-speed sedimentation assays to analyze binding to or the bundling of actin filaments (1). Low shear viscosimetry assays are being used to assess the viscosity shift upon actin polymerization in the presence or the absence of the compounds of interest (2). Electron microscopic analysis allowed the determination of filament elongation rates directly by measuring their lengths after elongation for a defined period as well as the effect of accessory proteins on filament architecture (1, 3, 4). More recently, the usage of fluorescently labeled phalloidin allowed direct visualization of filament annealing and severing (5, 6). However, because phalloidin stabilizes actin filaments, it is ineligible to monitor filament dynamics. Even though actin can also be labeled directly with fluorophores which only moderately affect actin polymerization, visualization of filament elongation by wide field microscopy has been hampered by the high background fluorescence caused primarily by the high amount of labeled actin monomers that is required to power actin assembly. In recent years, the study of actin dynamics in vitro has been dominated by the spectroscopic pyrenyl-actin assay (7), which allows to measure polymerization kinetics. One drawback of this otherwise highly effective assay is the difficulty to discriminate between an increased nucleation or elongation rate of filaments during polymerization. In other words, many nucleating filaments that elongate slowly may give the same fluorescence signal as a few rapidly elongating filaments. Finally, although most ensemble measurements such as pyrenyl-actin assays are expedient, convenient, and fast, they have the great disadvantage of not monitoring individual filaments. In 2001 Amann and Pollard (8) overcame these problems and developed an assay to follow polymerization dynamics and filament topology simultaneously. They combined the usage of partially labeled rhodamine-actin bound to *N*-Ethylmaleimide (NEM) myosin on the surface of flow cells with total internal reflection fluorescence (TIRF) microscopy (8), a method initially developed by Daniel Axelrod in the early 1980s (9).

1.1. The Principle of TIRF Microscopy

TIRF microscopy is an optical technique that allows the selective excitation of fluorophores within a small region above a glass coverslip, thus largely avoiding unwanted background fluorescence. In a TIRF microscope, a laser beam passes a high refractive index medium (mostly a special immersion oil) to a lower refractive index medium (mostly an aqueous solution) (Fig. 1a). This laser light beam is obliquely incident upon the substrate liquid interface at an angle greater than the critical angle of reflection. At this angle the light beam is totally reflected by the interface, which causes no longer direct illumination of the sample. Since light cannot be reflected at an infinite thin layer, an electromagnetic “evanescent” wave penetrates into the lower refractive index medium. It propagates parallel to the surface with an exponentially decaying intensity, exciting only fluorophores that are within a range of less than 200 nm to the reflecting coverslip surface (9).

1.2. Using In Vitro TIRF Microscopy to Visualize Actin Polymerization

By the use of partially labeled monomeric actin in a viscous polymerization buffer, the nucleation and constant elongation of single actin filaments can be monitored and analyzed by time-lapse TIRF

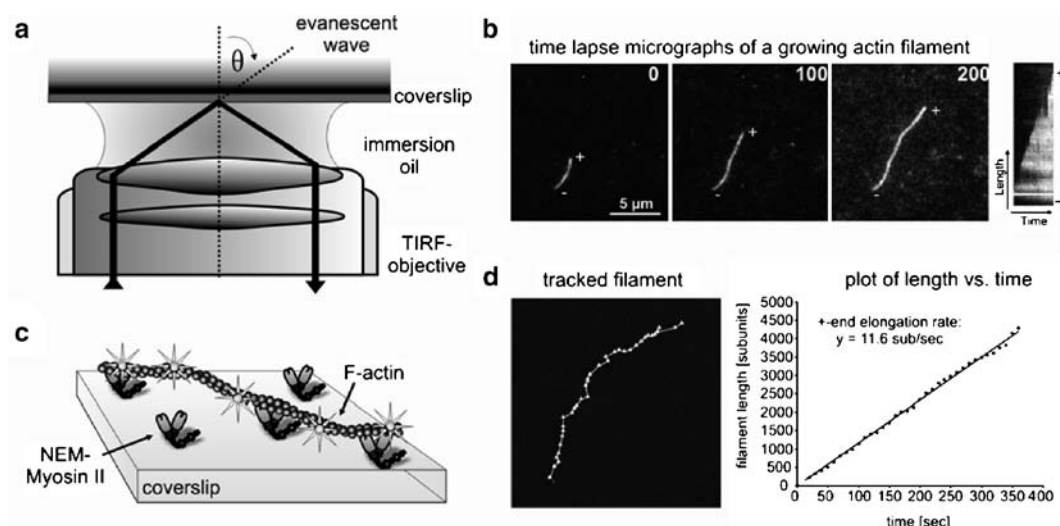


Fig. 1. In vitro TIRF microscopy of actin assembly (a) Principle of objective-based TIRF microscopy. Light from a laser excitation source passes through an objective with high numerical aperture and is reflected from the glass-buffer boundary at a critical angle θ , resulting in an evanescent wave above the coverslip that travels less than 200 nm. (b) Time-lapse micrographs of spontaneous filament assembly using 1.3 μM G-actin (30% labeled with Alexa-488- C_5 maleimide) in TIRF buffer on a NEM-myosin II coated coverslip. The kymograph (right) of the fluorescing filament reveals constant growth of the fast growing barbed (+) and slow growing pointed (–) ends. Time is shown in seconds. (c) Scheme of the visualization of single actin filaments using TIRF microscopy. Growing actin filaments attach to NEM-inactivated myosin II on the coverslip surface. Fluorophores within the filament in the evanescent wave on the coverslip are excited and emit secondary fluorescence, while fluorophores above are not excited. This way, single actin filaments can be monitored even in the presence of high amounts of fluorescently labeled G-actin. (d) Determination of the elongation rate of a single filament. The filament depicted in (c) was tracked using the imageJ-plugin MTrackJ (left) and the resulting overall lengths per frame were plotted over time (right). The elongation rate in subunits per second can be calculated from the slope of a simple linear regression assuming that 1 μm filament corresponds to 365 actin subunits.

microscopy when they are sufficiently close to the coverslip surface (**Fig. 1b**). This can be achieved by binding of the actin filaments to NEM inactivated myosin II, which in turn is immobilized on the glass coverslip (**Fig. 1c**). Covalent modification of myosin with NEM inhibits its motor activity, but still allows actin filament side-binding even in ATP-containing buffers (**10**). The growing ends of filaments can be tracked using image analysis software such as the freeware imageJ (<http://rsb.info.nih.gov/ij/>), and their elongation rates can be determined from time-lapse movies by measuring their length at consecutive time points. A plot of length versus time and subsequent linear regression of the slope yields the elongation rate of this particular actin filament (**Fig. 1d**). The great advantage of this method in comparison to bulk assays, such as the pyrenyl-actin assay, is that nucleation, elongation, bundling and branching of filaments can be unambiguously discriminated (**Fig. 2**). TIRF microscopy has already been successfully used to investigate the molecular details of formin-mediated actin assembly (**11–13**), the effects of PIP₂ on actin filament capping by capping proteins (**14**), filament branching by the Arp2/3-complex (**15**), the filament severing-activity of twinfilin and cofilin (**16–18**), and barbed end capture by VASP (**19**). In addition, the development and dynamics of more complex actin structures such as filament bundles and meshworks formed either by self-organization of actin (**20**), or induced by crosslinking proteins such as VASP (**21**) could be directly monitored and analyzed (**Fig. 3**).

1.3. TIRF Microscopy Reveals Novel Insights into the Function of VASP

Enabled/vasodilator-stimulated phosphoproteins (Ena/VASP) are a structurally conserved family found in vertebrates, invertebrates, and *Dictyostelium discoideum* cells. Ena/VASP proteins were shown

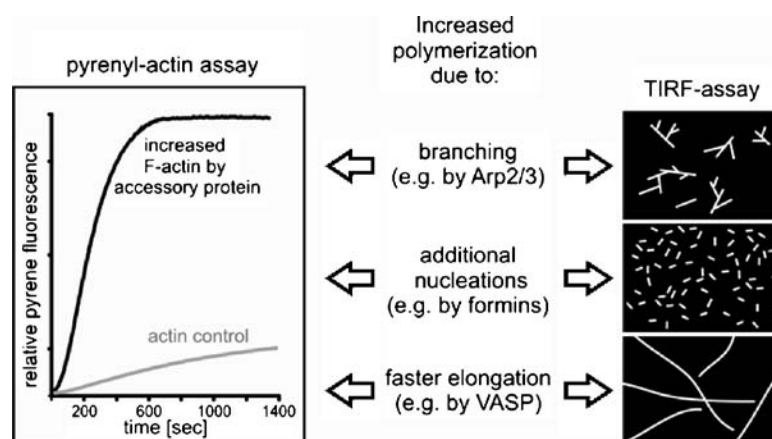


Fig. 2. Comparison of the pyrenyl-actin assay and in vitro TIRF microscopy as tools for the analysis of actin assembly. Actin-binding proteins have different effects on actin polymerization. Whereas the pyrenyl-actin assay does not allow to clearly distinguish between de novo nucleation (e.g., by formins), nucleation through branching on preexisting filaments (by Arp2/3-complex) or enhanced filament elongation (e.g., by VASP), these events can be unambiguously discriminated by in vitro TIRF microscopy.

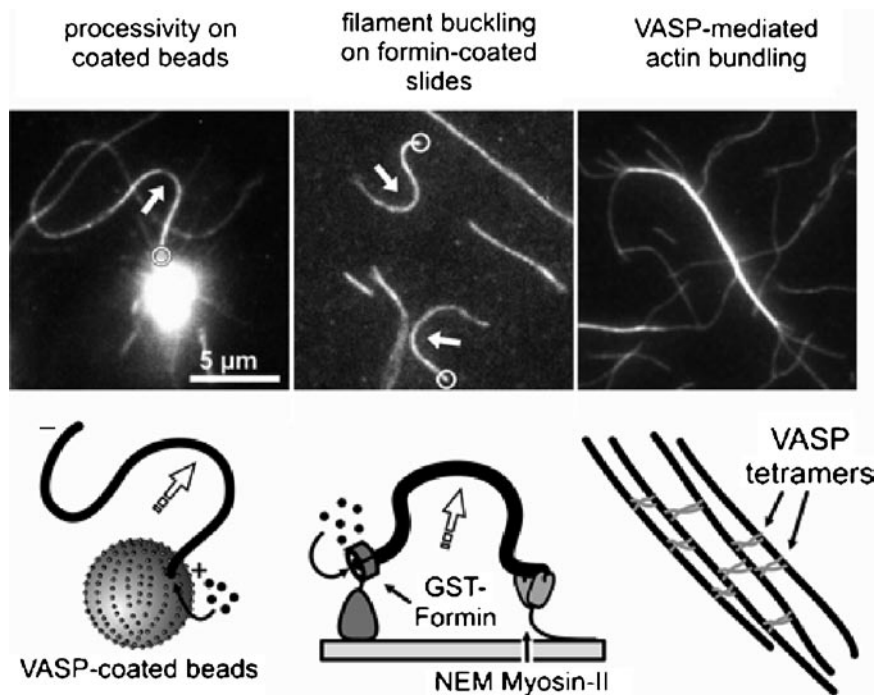


Fig. 3. In vitro TIRF microscopy can also be employed to visualize complex protein–actin interactions. By attaching F-actin elongating proteins on beads (e.g., VASP, *left*) or on coverslips (e.g., formins, *middle*), processive elongation of single filaments can be visualized and analyzed. In these cases, filament “buckling” (*white arrows*) can be observed due to the insertional assembly of actin monomers at the anchored filament barbed end (*white circles*). Moreover, the dynamic formation of complex structures such as filament bundles induced by VASP, fascin, or other actin-binding proteins in solution can be monitored in real time.

to be implicated in many actin-dependent processes including cell–cell adhesion, filopodia formation, and chemotaxis by *Dictyostelium* amoebae (22, 23). Although it was known for about a decade that Ena/VASP proteins tetramerize, bind directly to profilin as well as to both monomeric and filamentous actin, and are able to nucleate and bundle actin filaments in vitro (24), there was considerable controversy in the field concerning the precise mode of action of these remarkable actin-binding proteins (25). By analyzing single filament dynamics using TIRF microscopy we were able to show that VASP actively accelerates filament elongation by delivering actin monomers to the growing barbed end by employing its WH2 domain-related actin-binding sites (21). Moreover, we found that whereas in solution, VASP markedly accelerates actin filament elongation in a concentration-dependent manner but is inhibited by low concentrations of heterodimeric capping protein, clustering of VASP on functionalized beads induces processive filament elongation that becomes virtually insensitive to capping protein. Thus, like formins, VASP is capable of tethering filaments to a surface while adding subunits to the barbed end, although utilizing an entirely different mechanism.

2. Materials

2.1. Preparation and Modification of Actin and Myosin from Rabbit Skeletal Muscle

2.1.1. Preparation of Actin from Rabbit Skeletal Muscle

Keep all instruments, buffers and materials at 4°C.

1. 1.5 kg freshly prepared rabbit muscle.
2. Sharp knife or scalpel.
3. Meat grinder.
4. 5-L glass or plastic beaker.
5. Extraction buffer 1 (2.5 L): 0.5 M KCl, 0.1 M K_2HPO_4 , 0.05 M KH_2PO_4 , pH 6.4.
6. Extraction buffer 2 (2.5 L): 0.5 M KCl, 0.1 M K_2HPO_4 , 0.05 M KH_2PO_4 , 0.5 mM ATP, pH 6.4.
7. 1 M Na_2CO_3 (10 mL).
8. 5 L acetone at 4°C.
9. Aluminum foil.

2.1.2. Preparation of G-actin from Acetone Powder

1. Gauze.
2. Rubber band.
3. 250-mL glass beaker.
4. G-actin buffer (G-buffer) (2×, 5 L): 2 mM Tris-HCl of pH 8.0, 0.2 mM CaCl_2 , 0.2 mM ATP, 0.01% NaN_3 , 0.5 mM DTT.
5. Actin polymerization buffer (50×): 2.5 M KCl, 100 mM MgCl_2 , 50 mM ATP.
6. 10/300 G-75 gel filtration column (GE Healthcare).

2.1.3. Labeling of Actin with Alexa-Fluor-Maleimide Dyes

1. Alexa-Fluor-488- C_5 -maleimide (Molecular Probes).
2. G-buffer (*see Subheading 2.1.2*).
3. Labeling buffer (P-buffer): 1 mM NaHCO_3 , pH 7.5 (adjusted with Na_2CO_3), 0.1 mM CaCl_2 , 0.2 mM ATP.
4. Water-free dimethylsulfoxide (DMSO).

2.1.4. Preparation of NEM-Heavy-Mero-Myosin II (NEM-HMM)

1. Several liters of 4°C water.
2. High-salt buffer (0.5 L): 0.1 M K_2HPO_4 , 0.05 M KH_2PO_4 of pH 7.6, 0.5 M KCl, 0.1 mM EGTA, 1 mM DTT.
3. Glycerol.
4. α -chymotrypsine buffer (CHB-buffer) (2×): 20 mM imidazole of pH 7.0, 1 M KCl, 4 mM MgCl_2 , 10 mM DTT.
5. Buffered EGTA-DTT (BED): 0.1 mM NaHCO_3 of pH 7.0, 0.1 mM EGTA, 1 mM DTT.
6. Tosyl-l-lysine chloromethyl ketone (TLCK) treated α -chymotrypsine.
7. 1 M MgCl_2 solution.

8. PMSF (4 mg/mL in methanol).
9. *N*-Ethylmaleimide (NEM).
10. CHB (1×): 1 volume 2× CHB + 1 volume BED.

2.2. Purification of GST Fusion Proteins

1. LB-rich growth medium: 5 g/L NaCl, 8 g/L yeast extract, 16 g/L tryptone/peptone, 10 mM potassium phosphate buffer, pH 7.3.
2. Ampicillin stock solution (1,000×): 0.5 g ampicillin; bring to 10 mL with deionized water, sterile filter. Store at 4°C.
3. 1 M IPTG stock solution in ddH₂O, sterile filter. Store at -20°C.
4. PBS: 2.7 mM KCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 140 mM NaCl, pH 7.3.
5. Lysis buffer: PBS containing 5 mM benzamidine, 1 mM EDTA, 3 mM 2-mercaptoethanol or 1 mM DTT, 5 mM PMSF.
6. Elution buffer: 30 mM reduced glutathione in lysis buffer; readjust pH with 1 M NaOH to pH 7.3.
7. Glutathione sepharose 4B (GE Healthcare) or Glutathione agarose (Sigma).

2.3. TIRF Microscopy

2.3.1. Microscopic Setup

To observe the dynamics of small structures such as the growth of single actin filaments in solution or on functionalized beads, a specialized TIRF microscope is needed. Critical for TIRF imaging are furthermore a sensitive CCD Camera, high-quality objectives with a high numeric aperture ($NA > 1.4$), immersion oil (e.g., Leica with a refractive index of 1.518) as well as suitable lasers and filter sets. These days, complete systems for TIRF microscopy can be purchased from manufacturers such as Olympus, Nikon or Leica.

2.3.2. Preparation of Flow Cells

1. 24 × 40 × 0.17 mm glass coverslips.
2. 76 × 26 mm glass slides.
3. Thin double-faced adhesive tape or Parafilm.
4. Ultrasonic bath.
5. Rinsing agent.
6. 70% and 96% ethanol.
7. 1 M EDTA solution, pH 7.0.
8. 1 M HCl.
9. 1 M KOH.

2.3.3. Actin Polymerization Assays

1. Suitable TIRF microscope (e.g., an inverted Olympus IX71 equipped with an PLAPON 60XOTIRFM objective with 1.45 NA, adequate filter sets, a laser emitting 20–50 mW at 488 nm wavelength, and a high-resolution CCD camera such as Hamamatsu ER C8484).

2. Appropriate flow cells (**Subheading 3.3.1**).
3. TIRF buffer (2×): 20 mM imidazole, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 0.4 mM ATP, 100 mM DTT, 30 mM glucose, 40 µg/mL catalase, 200 µg/mL glucose oxidase, and 1% methylcellulose (4,000 centipoise, cP).
4. High-salt TBS (HS-TBS) (2×): 100 mM Tris-HCl of pH 7.6, 1.2 M NaCl.
5. Low-salt TBS (LS-TBS) (2×): 100 mM Tris-HCl of pH 7.6, 100 mM NaCl.
6. 1% bovine serum albumin (BSA) in HS-TBS and LS-TBS.
7. Antibleach solution: 1 mg/mL catalase, 5 mg/mL glucose oxidase in TIRF buffer.
8. Dust-free ddH₂O.

3. Methods

3.1. Preparation and Labeling of Actin and Myosin from Rabbit Skeletal Muscle

3.1.1. Preparation of Acetone Powder from Rabbit Skeletal Muscle

1. Prepare everything at 4°C.
2. Cut up 1.5 kg of fresh rabbit muscle meat, remove fat generously, and turn meat twice by a meat grinder.
3. Stir the ground meat for 15 min after addition of 2.5 L extraction buffer 1 with a solid glass rod and subsequently centrifuge the slurry at 4,000 × g for 7 min.
4. Remove but keep the supernatant for preparation of myosin II (**Subheading 3.1.4**).
5. Resuspend the pellet with 2.5 L extraction buffer 2, stir the slurry again for 15 min, and centrifuge at 4,000 × g for 7 min.
6. Resuspend the pellet in 2.5 L of cold ddH₂O and adjust the pH to 8.3 with 1 M Na₂CO₃ and stir for 10 min. Subsequently, pellet the slurry at 4,000 × g for 7 min.
7. Repeat this step two to three times until a recognizable swelling of the pellet occurs (the slurry will also become transparent). This step is critical since washing too long will result in considerable loss of G-actin.
8. Subsequently, resuspend the pellet in 4°C cold acetone, stir the slurry for 15 min, and centrifuge again for 7 min at 4,000 × g.
9. Discard the supernatant and repeat this step one more time.
10. Discard the supernatant, break up the pellet with your hands into small pieces over a sufficiently large piece of aluminum foil, and allow the acetone powder to dry under a fume hood at room temperature over night.

11. Fill the desiccated acetone powder in 50-mL reaction tubes, close the caps tightly, and store the tubes below -20°C . 1.5 kg of rabbit muscle will yield approximately 100 g of acetone powder.

3.1.2. Preparation of G-actin from Acetone Powder

1. Prepare everything at 4°C .
2. Soak 10 g of the acetone powder in 100 mL cold G-buffer for 15 min.
3. Filter the suspension through a gauze stringed with a rubber band over a 250-mL glass beaker.
4. Repeat the extraction up to five times and control the amount of extracted actin by SDS-PAGE and Coomassie Blue staining.
5. Pool the fractions containing most of the actin, and subsequently clear spin the suspension at $30,000 \times g$ for 30 min.
6. Polymerize actin in the supernatant by the addition of 50 $\times$ actin polymerization buffer to a final concentration of 1 $\times$ for at least 2 h at room temperature or at 4°C over night.
7. Subsequently, increase the KCl concentration in the viscous solution to 0.8 M.
8. Spin F-actin in an ultracentrifuge for 3 h at $150,000 \times g$.
9. Discard the supernatant. Resuspend and homogenize the hyaline pellet using a Dounce homogenizator with 15 mL G-buffer. Depolymerize F-actin by dialysis against G-buffer at 4°C with several buffer changes for at least 3 days.
10. Spin the actin solution in an ultracentrifuge for 3 h at $150,000 \times g$.
11. Take the upper two-third of the supernatant and separate monomers from di- and multimers by running the actin solution over a G-75 gel filtration column equilibrated in G-buffer.
12. Determine the actin concentration by measuring the OD at 290 nm ($\epsilon_{290} \text{ actin} = 26,600 \text{ M}^{-1}\text{cm}^{-1}$).

3.1.3. Labeling of Actin with Alexa-Fluor Maleimide Dyes

1. Prepare everything at 4°C and protect the Alexa dye as good as possible from incident light (*see* [Notes 1](#) and [2](#)).
2. Dialyze G-actin-containing supernatant from **step 11** of **Subheading 3.1.4** or purified G-actin against P-buffer with three buffer changes.
3. Resuspend 1 mg of Alexa-Fluor-488- C_5 -maleimide in water-free DMSO and store unused dye at -20°C .
4. Label G-actin by adding a twofold to fivefold molar excess of the fluorescent dye and stir the solution vigorously while adding the dye (*see* [Note 3](#)).
5. Stir slowly over night at 4°C in the dark.

6. Dialyze the solution against G-buffer containing 60% glycerol with three buffer changes. This will remove free dye, concentrate your protein, and allow you to store labeled actin at -20°C .
7. Before usage, dialyze an aliquot against three changes of G-buffer.
8. Measure absorbance of the supernatant at 280 nm and at 495 nm (ϵ_{280} Alexa-488-actin = $1.09 \times 42,000 \text{ M}^{-1}\text{cm}^{-1}$, ϵ_{495} Alexa-488 = $71,000 \text{ M}^{-1}\text{cm}^{-1}$).
9. The concentration of Alexa-labeled actin can be determined by the equation: $[\text{c labeled actin}] = \text{abs at } 290 \text{ nm} - 0.1377 \times \text{abs at } 488 \text{ nm}$.

3.1.4. Preparation of NEM-Heavy-Mero- Myosin II (NEM-HMM)

1. Dilute the supernatant from **Subheading 3.1.4** with 18 volumes of 4°C cold ddH_2O and incubate over night. This will cause the myosin II to precipitate.
2. Spin the solution at $5,000 \times g$ for 40 min and resuspend the pellet in 500 mL high-salt buffer.
3. Remove actin by centrifugation at $30,000 \times g$ for 40 min and filter the supernatant over gauze to remove the lipid layer.
4. Repeat the precipitation of myosin II by adding 10 volumes of ddH_2O and proceed as described in **steps 2 and 3**.
5. Resuspend the pellet in 200 mL high-salt buffer containing 55% glycerol. This solution can be stored at -20°C for several months.
6. Dilute 3 mL of the prepared myosin II solution in 27 mL of cold BED-buffer and incubate for 10 min on ice.
7. Spin the myosin with $9,000 \times g$ for 10 min at 4°C .
8. Resuspend the pellet with 0.5 mL $2\times$ CHB-buffer lacking DTT.
9. Determine the concentration of the protein ($\epsilon_{280} = 0.53 \text{ mL} \times \text{mg}^{-1}\text{cm}^{-1}$), add $1\times$ CHB-buffer with DTT to a final concentration of 15 mg/mL, and incubate 10 min at 25°C .
10. Add $12.5 \mu\text{g/mL}$ TLCK-treated α -chymotrypsine and incubate 8 min at 25°C .
11. Stop the reaction by adding 4.5 mL of cold BED-buffer containing 3 mM MgCl_2 and 0.1 mM PMSF.
12. Incubate 1 h on ice and remove the cleaved insoluble tail fragments of myosin II by centrifugation at $150,000 \times g$ for 15 min at 4°C .
13. Determine the concentration of HMM II in the supernatant ($\epsilon_{280} = 0.6 \text{ mL} \times \text{mg}^{-1}\text{cm}^{-1}$). Optionally, you can concentrate the myosin using spin-columns or ultrafiltration.
14. To inactivate HMM with NEM, incubate 1 mg/mL HMM with 0.3 mM NEM for 1 h at room temperature.
15. To remove NEM, dialyze against BED-buffer over night at 4°C .

3.2. Purification of GST-DdVASP

16. Add sucrose to a final concentration of 30% and shock-freeze the protein solution in 50 μ L aliquots using thin-walled 200- μ L reaction tubes in liquid nitrogen. Store at -80°C .
1. The purification of GST-tagged DdVASP is described here exemplarily. The purification of other actin-binding proteins fused to GFP can be performed accordingly.
 2. For the expression of GST-tagged DdVASP, previously designed plasmid pGEX-5x-1-DdVASP was used (23).
 3. Transform the expression plasmid in competent *E. coli* BL21 cells and check expression of GST-DdVASP by small-scale induction of individual clones.
 4. Inoculate 200 mL of LB-rich medium supplemented with 50 $\mu\text{g/mL}$ of ampicillin with an expressing clone and grow the preculture over night at 37°C while shaking at 200 rpm to stationary phase.
 5. Dilute 20 mL of the preculture into 1 L of LB-rich medium containing 50 $\mu\text{g/mL}$ of ampicillin in a 2-L Erlenmeyer flask with baffles and grow the cells at 25°C while shaking at 200 rpm until an $\text{OD}_{600\text{nm}}$ of 0.5 is reached.
 6. To induce expression of GST-tagged DdVASP add IPTG to a final concentration of 0.5 mM.
 7. Continue to grow the culture while shaking over night at 23°C at 200 rpm.
 8. Harvest the cells by centrifugation in a prechilled centrifuge for 20 min at 4°C and $8,000 \times g$. Discard the supernatant and weight the pellet. All following steps should be carried out on ice or below 4°C to prevent degradation of the fusion protein.
 9. Resuspend the pellet in $\sim 2 \text{ g/mL}$ of lysis buffer, add lysozyme to a final concentration of 2 mg/mL and incubate for 15 min on ice.
 10. Sonicate the resuspended cells in an ice-water bath with a Branson sonifier 250 (or similar) during three 2-min intervals using a 60% duty cycle and a 400-W output.
 11. Spin the lysate for 60 min at $35,000 \times g$ in a Beckman Avanti J-30I centrifuge (or similar) at 4°C and keep the supernatant.
 12. While the lysate is centrifuging, add 10 mL of glutathione sepharose slurry to a Biorad Econo column (or similar) and equilibrate the column with at least two column volumes of lysis buffer.
 13. Load the supernatant on the column, using a peristaltic pump at a flow rate of $\sim 0.5 \text{ mL/min}$ and pass the flow-through onto the column at least two more times.
 14. After loading, wash the column with at least 100 mL of lysis buffer.

15. Elute bound protein with 10–50 mL of elution buffer at a maximum flow rate of 1 mL/min and collect 4 mL fractions using a fraction collector or manually (*see* [Note 4](#)).
16. To determine the protein concentration and purity, check a sample of each fraction in a Bradford assay (or in a photometer at 280 nm absorption) and by SDS-PAGE followed by Coomassie blue staining.

3.3. TIRF Microscopy

3.3.1. Preparation of Flow Cells

1. Place coverslips into a suitable rigid polyvinyl chloride or glass rack.
2. Add 60°C warm ddH₂O with 2% rinsing agent.
3. Sonicate for 45 min.
4. Rinse thoroughly with ddH₂O to remove the detergent.
5. Incubate for 3 h at 40°C in 1 M KOH while stirring.
6. Incubate over night at 40°C in 1 M HCl while stirring.
7. Rinse thoroughly with ddH₂O.
8. Sonicate in dust-free ddH₂O for 30 min and add 10 mM EDTA after 15 min.
9. Rinse thoroughly with ddH₂O.
10. Sonicate subsequently in dust-free 70% ethanol followed by sonication in 96% ethanol.
11. Rinse thoroughly with dust-free 60°C ddH₂O and immediately dry the coverslips under the air flow of a clean bench.
12. Prepare glass slides with two small stripes of double-faced adhesive tape or Parafilm. The spacing between the stripes should be ~4–10 mm.
13. Use tweezers to carefully place the clean and completely dried coverslips onto the stripes. Compress the stripes and the coverslips firmly to seal the sides of the flow cell. When using Parafilm, use a flame to moderately melt the Parafilm and gently press the coverslip onto it afterward (**Fig. 4**).
14. Store the flow cells dry and dust-free at room temperature.

3.3.2. Actin Polymerization Assays

It is absolutely crucial to use monomeric actin in the polymerization assay. Even the presence of very small actin multimers will drastically increase the number of growing filaments and therefore adulterate the analysis. We highly recommend gel filtration of actin using a 10/300 G-75 column (GE Healthcare) just prior to the experiment. Gel-filtered actin can be used for TIRF polymerization assays for at least 2 weeks when stored at 4°C in G-buffer.

1. Prepare the reaction mixture (usually 100 µL) in the following order: x µL ddH₂O (depending on the concentrations of actin and the accessory proteins you use), 50 µL 2× TIRF buffer, 2 µL antibleach solution, and x µL of the accessory protein. Keep the mixture on ice (*see* [Note 5](#)).

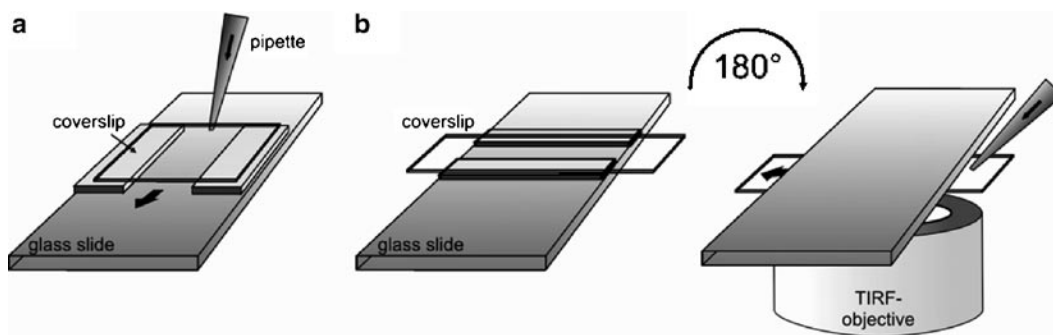


Fig. 4. Scheme of two different types of flow cells. The standard flow cell consists of a coverslip attached in parallel orientation to the glass slide (*left*). Due to the configuration of an inverted microscope resulting in restricted spatial access to the vents of chamber once it is mounted on the stage, this flow cell is not suited to change their content during the measurement. Attachment of the coverslip perpendicular to the axis of the glass slide instead allows the replacement of the reaction mixture during the measurement (*right*). This setup is instrumental to analyze dynamic changes of particular filaments induced by the infusion of additional proteins.

2. Prepare flow cell by adding the quantity required of NEM-inactivated HMM in HS-TBS. Incubate for 5 min (*see* [Note 6](#)) (Refer to **Subheading 3.1.4** for preparation of this reagent.).
3. Wash the flow cell by applying 50 μ L 1% BSA in HS-TBS and 50 μ L 1% BSA in LS-TBS on one side of the chamber and concomitantly use an absorptive cloth on the other side to pull it through the flow cell.
4. Equilibrate with 1 $\times$ TIRF buffer.
5. Mix labeled and unlabeled G-actin to the desired fraction (usually 20–30% labeling) (Refer to **Subheadings 3.1.2** and **3.1.3** for preparation of these reagents).
6. Add the reaction mixture to the G-actin solution. Mix thoroughly and prevent air bubbles in the solution. Load 50 μ L of the reaction mixture into the flow cell, add a droplet of immersion oil, and place it onto the microscope stage with the coverslip down facing the objective lens and start recording actin assembly.

4. Notes

1. The choice of the fluorescent dye used for labeling strongly affects the information that can be derived from polymerization experiments. Some dyes – like Alexa-488-maleimide – inhibit the interaction between actin and profilin or other G-actin-binding proteins and therefore change fluorescent intensities of the filaments when assembled by formins ([11](#)), an effect very useful to discriminate different filament species.

2. Depending on their resistance to photobleaching, labeling actin with different fluorescent dyes results in filaments that grow in a “comet like” appearance (e.g., Alexa-532; (12)) or in filaments that are almost resistant to bleaching (e.g., Oregon Green; (14)). Depending on the desired information, both effects can be very useful.
3. Since fluorescent dyes are rather expensive, it is useful to test different conditions like temperature, incubation time, and buffer composition for the labeling reaction at small scale. In any circumstance for labeling of cysteine residues DTT and azide should be avoided. For the labeling of lysine residues primary amines must be avoided, and it has proved useful to polymerize actin before labeling to avoid modification of residues involved in polymerization.
4. The addition of 30 mM of reduced glutathione drastically lowers the pH of the elution buffer from pH 7.3 to a pH of ~3.5. Since these conditions will denature most proteins, it is very important to readjust the solution to pH 7.3 with 1 M NaOH.
5. For detailed analyses of polymerization kinetics, PBS buffer should be avoided or at least kept at a constant concentration in the experiments, since phosphate alters the polymerization- and ATP-hydrolysis kinetics of actin (26).
6. It is not recommended to “glue” the filaments to the coverslip by using too much of NEM-HMM. The less myosin is used, the better is the image quality and the more natural is actin filament growth and structure. Furthermore, the extreme slow growth of the filament (–) ends is only observable with low amounts of NEM-HMM.

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Chapter 28

Single-Molecule Imaging Techniques to Visualize Chemotactic Signaling Events on the Membrane of Living *Dictyostelium* Cells

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Summary

In this chapter, we describe methods to monitor signaling events at the single-molecule level on the membrane of living cells by using total internal reflection fluorescence microscopy (TIRFM). The techniques provide a powerful tool for elucidating the stochastic properties of signaling molecules involved in chemotaxis of the cellular slime mold *Dictyostelium discoideum*. Taking cAMP receptor 1 (cAR1) as an example of a target protein for single-molecule imaging, we describe the experimental setup of TIRFM, a method for labeling cAR1 with a fluorescent dye, and a method for investigating the receptor's lateral mobility. We discuss how the developmental progression of cells modulates both cAR1 behavior and the phenotypic variability in cAR1 mobility for different cell populations.

Key words: TIRFM, Single molecule, cAR1, Lifetime Diffusion, Lateral mobility

1. Introduction

Intracellular signal transduction is a stochastic process because it depends on chemical and physical reactions such as the association/dissociation, enzymatic catalysis, chemical modification and diffusion of signaling molecules. These signaling reactions take place under the strong influence of thermal fluctuations and stochastic noises. Single-molecule imaging analysis of chemotactic signaling molecules in living *Dictyostelium* cells has identified the stochastic nature of the signaling molecules. For example, single-molecule imaging of a chemoattractant cAMP bound to the cAMP receptor demonstrates that signal inputs fluctuate with time and

space (1, 2). Downstream signaling molecules, such as PTEN and a PH domain-containing protein, that are constituent parts of the chemotactic signaling system can also be followed at the single-molecule level in living cells and therefore can illuminate the chemotactic signaling processes that accompany stochastic noise (3, 4). Because eukaryotic cells can exhibit chemotaxis even when the input signal intensities are approximately at the level of noise (5–8), a fundamental question regarding the intracellular chemotactic signaling processes arises. How does the signaling system operate reliably under thermal and stochastic fluctuations? Single-molecule imaging techniques provide a powerful tool to obtain information on how signals are received, processed, and transduced by stochastically operating molecules.

This chapter includes methods for visualizing single molecules that function on the membrane of living *Dictyostelium* cells. We first describe the instrumentation for objective-type total internal reflection fluorescence microscopy (TIRFM), followed by the method for single-molecule imaging by using TIRFM and a new labeling technique by using HaloTag® technology. Here we take cAMP receptor 1 (cAR1) from *Dictyostelium* as an example of a target protein for single-molecule imaging. The techniques described here can be applied to other signaling molecules such as G proteins. Single-molecule imaging techniques for the fluorescent cAMP analog, PTEN, and a PH domain-containing protein in living *Dictyostelium* cells have been described elsewhere (9, 10).

2. Materials

2.1. Experimental Setup of TIRFM for Single-Molecule Imaging

1. Components of TIRFM are shown in **Table 1**.
2. Software for image analysis, e.g., Image J (public domain), Image-Pro (Media Cybernetics, Maryland), and G-Track (G-Angstrom, Sendai, Japan).

2.2. Cell Preparation

1. Desalted deep sea water (DSW) (Marin Gold Corp., Muroto City, Kochi, Japan).
2. HL5 medium: 30.8 g of glucose, 14.3 g of yeast extract, 28.6 g of Proteose peptone (Difco), 0.97 g of KH_2PO_4 , 2.56 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.4 mg of folic acid, 0.12 mg of cyanocobalamin. Dissolve the ingredients in 2 L of DSW and sterilize by autoclaving.
3. Low-osmotic-strength buffer: 0.97 g of KH_2PO_4 , 2.56 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. Dissolve the ingredients in 2 L of H_2O and sterilize by autoclaving.

Table 1
Components of TIRFM

Microscope system feature	Examples of equipment	Manufacturer
Inverted microscope stand	IX71	Olympus (Tokyo)
Vibration isolation table	TDI high-performance three-dimensional six-degree-of-freedom vibration isolation system	HERZ (Yokohama, Japan)
Ultrasensitive camera	1M-Pixel IMPACTRON™ CCD camera	Texas instruments (Dallas, Texas)
Image intensifier	GaAsP image intensifier unit C8600 series	Hamamatsu photonics (Hamamatsu, Japan)
Excitation light source	Compass™ 315M series	Coherent (Santa Clara, California)
High-NA objective	PlanApo 60× N.A.: 1.45 oil	Olympus
Dichroic mirror	BrightLine® series (e.g., FF5–62-Di02, for TMR observation)	Semrock (Rochester, New York)
Bandpass filter	BrightLine series (e.g., FF01–593/40, for TMR observation)	Semrock
Beam expander	LBED	Sigma Koki (Tokyo)
Quarter wave plate	WPQ series	Sigma Koki
Other optics (neutral density filter, mirror and lens)		Sigma Koki

4. Development buffer (DB): 5 mM KH_2PO_4 , 5 mM Na_2HPO_4 , 2 mM MgSO_4 , 0.2 mM CaCl_2 . Dissolve the ingredients excluding MgSO_4 and CaCl_2 in H_2O and adjust the pH to 6.4. Sterilize by autoclaving. Then add MgSO_4 and CaCl_2 solutions which are dissolved in H_2O as concentration of 2 M and autoclaved as separate solutions.
5. Imaging buffer (IB): 5 mM KH_2PO_4 , 5 mM Na_2HPO_4 . Dissolve the ingredients and adjust the pH to 6.4. Sterilize by autoclaving.
6. Dithiothreitol (DTT): Prepare 2.5 M DTT stock in H_2O .
7. Electroporation buffer (EB): 10 mM NaPO_4 of pH 6.1, 50 mM sucrose. Dissolve the ingredients in H_2O , adjust the pH, and sterilize by passing the solution through a filter (pore size, 0.23 μm).
8. Square pulse electroporator: ECM 830 (BTX).

9. 2 mm gapped BTX electroporation cuvettes Plus™ (BTX).
10. Healing buffer: 100 mM CaCl₂, 100 mM MgCl₂. Dissolve the ingredients in H₂O. Sterilize by autoclaving.
11. cAMP for pulse stimulation: 10 μM cAMP in DB.
12. HaloTag TMR ligand (Promega, Madison, Wisconsin).
13. Caffeine: 50 mM caffeine in H₂O.

2.3. Visualizing Single Molecules Under TIRFM

1. Polyethylenimine (PEI): 2 μg/mL PEI in H₂O.
2. Attofluor® cell chamber (Molecular Probes, Eugene, Oregon).
3. Angle measure.

3. Methods

3.1. Experimental Setup of TIRFM

TIRFM can be used to observe fluorophores on the basal membrane of living cells near a glass surface. To illuminate fluorophores selectively near a glass surface by the excitation lights, TIRFM utilizes evanescent fields generated at the interface between the glass surface and aqueous solution. Theory of TIRFM has been described previously ([11](#)). When the excitation light for fluorophores is incident above some “critical angle” upon the glass/water interface, the light is totally internally reflected and generates a thin electromagnetic field, so-called evanescent fields, in the water (**Fig. 1a**). Careful adjustment of the angle of the incident light is important for successful setup of TIRFM.

1. **Figure 1b, c** illustrates the optical configuration of objective-type TIRFM on an inverted microscope (*see Note 1*). An objective lens with high numerical aperture (>1.4) is mounted on the inverted microscope (*see Note 2*). A laser beam is passed through a neutral density filter and a beam expander to adjust its power and diameter. In order to convert the polarization of the beam from linear to circular, we used a quarter-wave plate. The incident laser beam is focused on the back focal plane (BFP) of the objective (*see Note 3*). To perform single-molecule imaging, a laser beam is incident to the specimen at a power of ~1 mW on a circular area 30 μm in diameter.
2. The objective-type TIRFM is based on the fact that a laser beam off-axis at the BFP of the objective leaves the objective lens with an angle. When the incident beam is focused at the center of the objective on-axis, the microscope can be used as a standard EPI-fluorescence microscope. When the path is shifted from the center to the edge off-axis (i.e., between

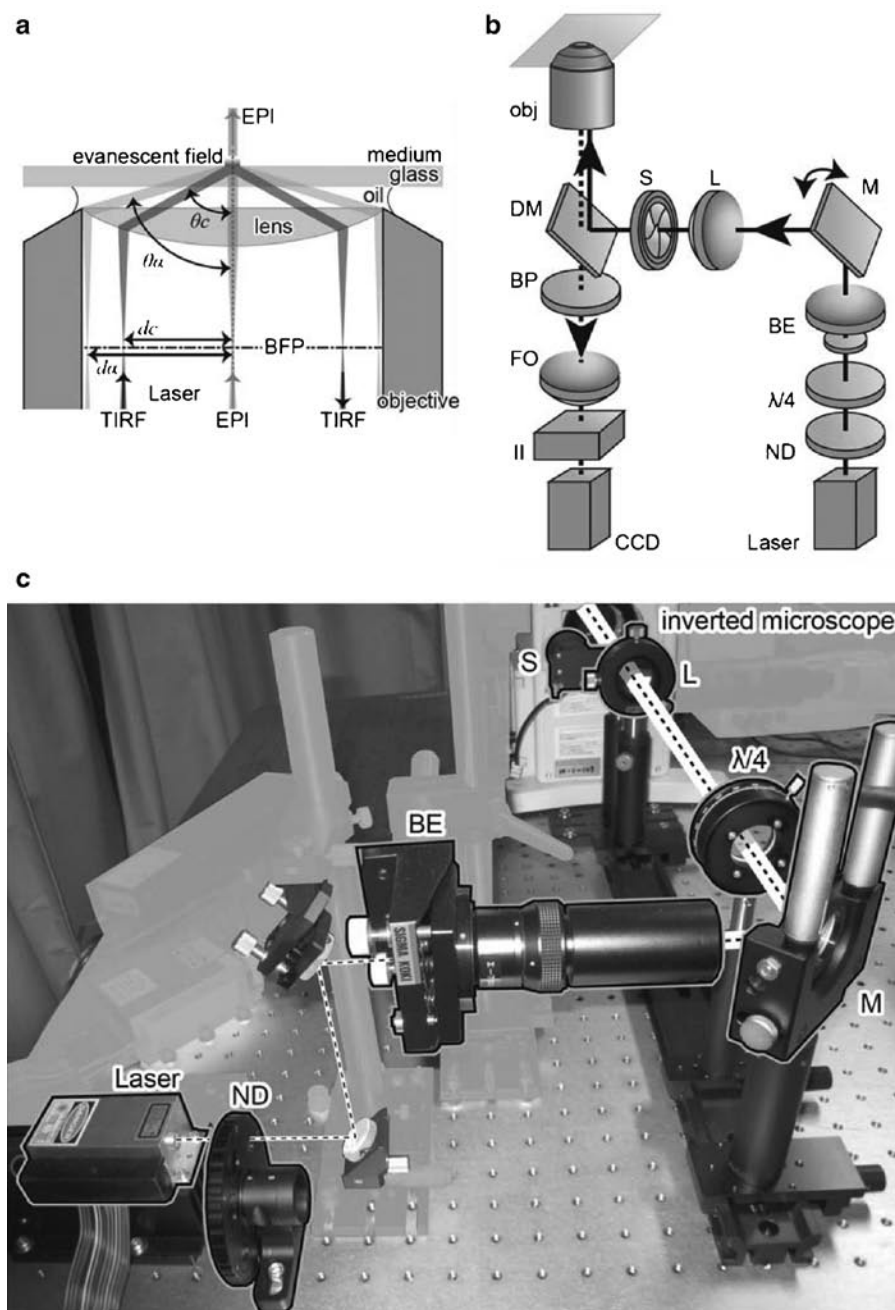


Fig. 1. Schematic drawing of objective-type TIRFM. (a) Light path of incident laser. The laser beam is focused on the back focal plane (BFP) of the objective lens. θ_c is the critical angle of the glass-water interface. θ_α is defined as $NA = n \sin \theta_\alpha$, where n is the refractive index of the glass and NA is the numerical aperture of the objective. When the incident beam is positioned at the objective edge between $d\alpha$ and dc , the beam is totally internally reflected, generating an evanescent field at the glass surface. Illumination mode can be switched from TIR to standard EPI by shifting the position of the beam focus at BFP from the edge to the center. (b) Configuration of objective-type TIRFM. ND neutral density filter; BE beam expander; $\lambda/4$ quarter-wave plate; M mirror; L focusing lens; S shutter; DM dichroic mirror; obj objective lens; BP band pass filter; FO focusing optics; II image intensifier; CCD charge-coupled device camera. Switching between epifluorescence microscopy and TIRFM can be performed by adjusting the position of a single mirror (M), which is located at the focus of the lens (L). (c) Photo of emission path used for TIRFM.

$d\alpha$ and dc , in **Fig. 1a**), the laser beam is incident above the critical angle θ_c at the glass/water interface where the beam is totally internally reflected, which generates evanescent fields in the water. Thus, the illumination of the excitation light can be switched from standard EPI to TIR simply by shifting the position of the beam focus at the BFP from center to edge. In our microscope, this shift is carried out by adjusting the position of the single mirror as shown in **Fig. 1b, c**.

3. Fluorescence signals from fluorophores are collected by the same objective. The scattered light from the incident laser beam is excluded by bandpass filters. The dichroic mirrors and filters should be carefully selected to maximize the specificity of fluorescence wavelength and to minimize the loss of fluorescence intensity.
4. The fluorescent images are acquired through an image intensifier by an ultrasensitive CCD camera. The images can be recorded to RAM in a PC with 33-ms intervals.

3.2. Cell Preparation

3.2.1. Culture and Development

Chemotactic responses by *Dictyostelium* to cAMP can be observed in developed cells. By starving in non-nutrient buffer, chemotactic competent cells can be obtained. The procedure is as follows.

1. Culture *Dictyostelium* cells in HL5 medium at 21°C with 100 µg/mL streptomycin sulfate and 100 U/mL benzylpenicillin potassium (**12**). When transformed cells are grown with selection, supplement HL5 medium with 20 µg/mL neomycin.
2. Harvest the cultured cells. Wash the cells with DB and resuspend them in DB to a cell density of 3×10^6 to 5×10^6 cells/mL.
3. Pour 1 mL of the cell suspension into a 35-mm Petri dish.
4. Keep the dish still for the first hour of incubation, and then move it to a shaker and add cAMP (100 nM final) every 6 min for the next few hours until the cells show polarized shapes, which is a hallmark of chemotactic competence. Proper development is critical to obtain reliable data of chemotactic signaling molecules in *Dictyostelium* cells (see **Note 4**). Note that some mutant cell lines have a defect in developmental progress, which affects cAR1 mobility (see **Note 5**).

3.2.2. Transformation of Cells

To transform cells with a plasmid vector carrying the gene of a target protein for single-molecule imaging, electroporation can be carried out by using a square pulse electroporator.

1. Suspend 5×10^7 cells in a 400 µL of EB. Just before electroporation, add 5 µg of the vector to the cell suspension and infuse into an electroporation cuvette (see **Note 5** and **6**).
2. Electroporate the cells under the following conditions: charging voltage, 500 V; width of pulse, 100 µs; number of square pulses, 15 with 1-s intervals (**13**).

3. Following electroporation, incubate the cell suspension at 21°C for 15 min with 4 μ L of healing buffer and then add the mixture of 10 mL of buffer with 10 mL of low-osmotic-strength buffer to the electroporated cell suspension (*see* [Note 7](#)).
4. Incubate the dishes for 24 h at 21°C followed by 20 μ L of 20 mg/mL geneticin.
5. Incubate the dishes for 1 week until the clones become detectable.

3.2.3. Labeling with HaloTag Ligand

Fluorescent labeling of a target protein by HaloTag technology is suitable for single-molecule imaging (*see* [Note 8](#) and [9](#)). Halo-tagged proteins are expressed in cells and then the proteins are labeled with fluorescent HaloTag ligands before observation.

1. Label Halo-tagged proteins by adding HaloTag TMR ligand (~50 nM final). Adjust the concentration of HaloTag TMR ligand if needed (*see* [Note 10](#)).
2. Incubate for 30 min at 21°C gently shaking. If needed, add caffeine to a final concentration of 4 mM (*see* [Note 11](#)).
3. To wash out unbound HaloTag TMR ligands, gently replace the TMR ligand-containing buffer with fresh IB twice, and then incubate for 10 min in IB. Repeat this washing procedure three times.
4. Suspend cells in IB and keep on ice until observation.

3.3. Visualizing Single Molecules Under TIRFM

1. Clean coverslips by ultrasonication in 0.1 M KOH for 30 min. Rinse in pure ethanol for 30 min. Repeat the rinse twice and store in pure ethanol. Cleaning of coverslips is critical for single-molecule imaging to remove nonspecific fluorescent dusts.
2. In order to facilitate the cell adhesion to the coverslip glass surface, coat the coverslips with PEI (*see* [Note 12](#)). Place 20 μ L of PEI onto the washed coverslips in a moist chamber. Wait 10 min and then wash the coverslip in a beaker containing ultra pure water. Dry the coverslips in a clean box.
3. Set the dry coverslip in an Attotfluor cell chamber. Place 10 μ L of the cell suspension onto the coverslip in the chamber. Wait several minutes for the cells to settle on the glass surface. Gently pour and fill the chamber with IB. Cell stimulation is achieved by adding cAMP solutions to the cell chamber. If needed, IB is supplemented with DTT at 10 mM to inhibit cAMP degradation.
4. Start up TIRFM (i.e., turn on the laser, the image intensifier, the CCD camera, etc.).
5. Adjust the incidence angle of the laser beam above the critical angle by adjusting the position of the mirror. The incidence angle can be measured using a thick glass slide of about 3 mm as illustrated in [Fig. 2](#). The incident beam is led to the glass

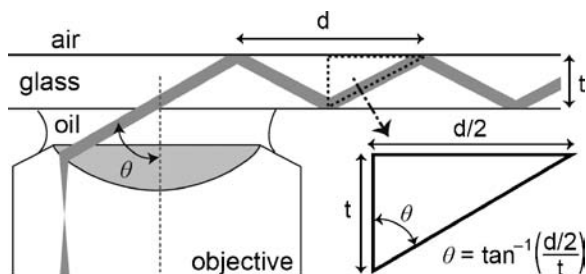


Fig. 2. Angle measurements of incident laser. The incident laser beam reaches the upper surface of the glass and reflects totally internally at the interface between the air and glass. The beam repeats the reflection. The incident angle can be calculated from the thickness of the glass (t) and the distance between the reflection spots (d) by taking the inverse tangent.

slide on the objective and totally internally reflected several times. By measuring the thickness of the glass slide and the intervals between the laser spots on the surface of glass slide using a caliper, the incidence angle can be determined (*see Note 13*).

6. Shut the laser by closing a shutter and then set the chamber on the stage of the TIRFM. Bring the specimen into focus by observing it under transmitted light illumination.
7. Turn off the transmitted light and open the shutter to guide the laser beam to the specimen. If the incidence angle attained is above the critical angle, the incident laser reflects totally internally at the interface between the glass coverslip and aqueous solution. The reflection beam is returned through the objective, which can be observed next to the incident beam at the lens or the mirror.
8. Initially observe the image by eye (i.e., without using the camera) using strong laser intensity. Focus on the fluorescent dyes attached to the glass surface or cell membrane, and adjust until they appear as clear spots.
9. Turn down the laser intensity. Acquire images through image intensifier and CCD camera.

3.4. Single-Molecule Tracking

The images obtained under TIRFM contain information about many individual molecules concomitantly (**Fig. 3a**). To obtain information for each single molecule, follow individual molecules to obtain their respective trajectories.

1. Track each single molecule by using appropriate software. We use G-Track for this purpose.
2. For lifetime analysis, collect the time duration between the appearance and disappearance of individual fluorescent spots on the membrane (**Fig. 3b**).

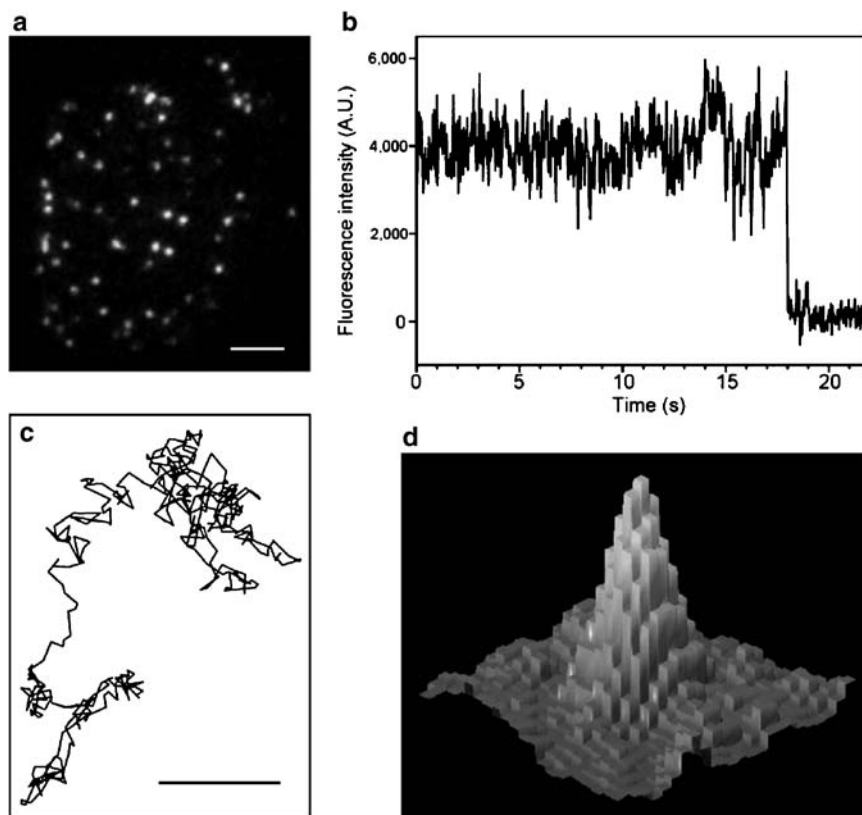


Fig. 3. Single-molecule imaging analysis of cAR1-Halo-TMR in living *Dictyostelium* cells. (a) Typical image of single molecules of cAR1-Halo-TMR. Bar, 2 μm . (b) Typical time course of fluorescence intensity of single cAR1-Halo-TMR spots showing single step photobleaching. (c) Examples of trajectories on cell membranes exhibited by single cAR1-Halo-TMR molecules. Bar, 200 nm. (d) Intensity profile of a fluorescent spot representing a single cAR1-Halo-TMR molecule.

3. For diffusion analysis, collect X-Y coordinate data of individual single molecules in each frame by determining the center of the fluorescent spot (Fig. 3c). The center position can be determined by calculating the center of the fluorescence intensities. Higher spatial resolution can be achieved when the intensity profiles of the fluorescence spots are fitted to the point spread function that has a Gaussian distribution (Fig. 3d).

3.5. Lifetime Analysis

Lifetimes of single molecules on the plasma membrane contain information about the fluorescence molecules (i.e., photobleaching) and target proteins (e.g., kinetics of dissociation from the membrane) (2, 3). Lifetime analysis has been successfully applied to the study of ligand-receptor complexes and downstream molecules including PTEN and PH domain-containing proteins (2–4).

1. Construct cumulative probability distributions of duration by counting the fraction of duration with values less than or equal to the corresponding time points. This probability distribution

reflects the release of molecules from the membrane. When transmembrane proteins such as receptors are observed, the probability distribution reflects the bleaching events of the fluorescence molecules attached to the proteins. Cumulative probability distributions of cAR1-Halo-TMR and cAR1-YFP are shown in **Fig. 4** (also *see* **Notes 14** and **15**).

2. Fit the probability distribution with the equation,

$$f(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + \dots$$

and calculate the time constants τ (*see* **Note 16**). In these equations, a_i represents the relative amounts of the i th component; that is the summation of a_i equals 1:

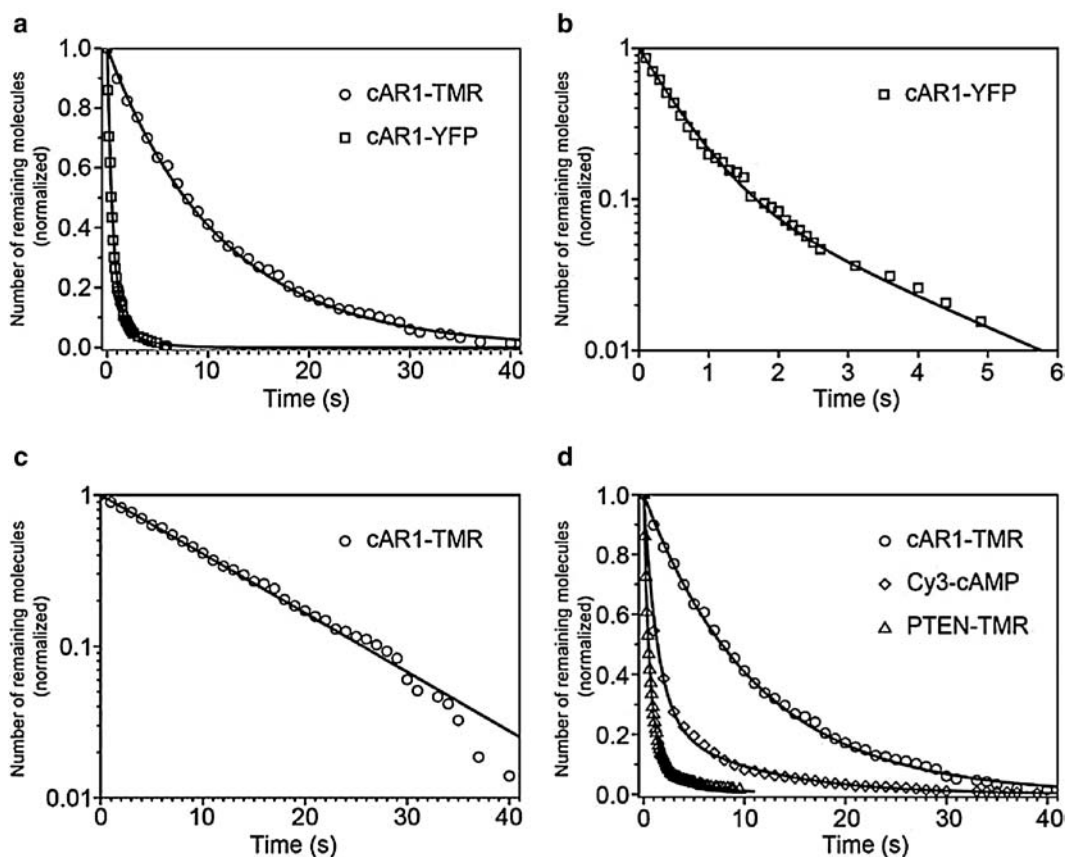


Fig. 4. Lifetime analysis of cAR1-Halo-TMR and cAR1-YFP. **(a)** Cumulative probability distributions of cAR1-TMR (circle) and cAR1-YFP (square). Solid lines represent the fitting of data to a single or sum of exponential functions. The time constant of cAR1-Halo-TMR, $\tau = 11$ s, represents the photobleaching time constant of TMR because cAR1 exists stably on the membrane. cAR1-YFP had two time constants, $\tau_1 = 0.52$ s (86%) and $\tau_2 = 2.2$ s (14%). This lifetime complexity for cAR1-YFP may be due to YFP blinking. Semilogarithmic plots of cumulative probability distributions of cAR1-YFP **(b)** and cAR1-Halo-TMR **(c)**, indicating multiple constants and single constant photobleaching time-lines, respectively. The semilogarithmic representation facilitates to discriminate the heterogeneity of lifetimes. **(d)** Lifetimes of cAR1-Halo-TMR, PTEN-Halo-TMR, and Cy3-cAMP.

$$\sum_{i=1}^N a_i = 1$$

Multiple dissociation time constants indicate the existence of multiple events occurring on the membrane. Fitting curves were superimposed in **Fig. 4**.

3.6. Short-Range Diffusion Coefficients Analysis

Short-range diffusion (SRD) analysis is the method used to obtain the distribution of diffusion coefficients of target molecules. In this method, short trajectories with constant time duration are successively extracted from one trajectory, and diffusion coefficients are determined for individual short trajectories, producing a time series of diffusion coefficients. This analysis can be used to detect temporal changes in the mobility of individual molecules ([14, 15](#)). In this section, we suppose that the images were acquired at 33-ms intervals (i.e., 30 frames per second (fps), $t_{\text{intvl}} = 33$ ms).

1. Extract all possible short trajectories with 500-ms duration from each trajectory. For example, successive 31 short trajectories can be derived from a trajectory of 1.5 s when the images were acquired at 30 fps.
2. Make a mean-squared displacement (MSD) versus lag-time (Δt) plots for each short trajectory. The squared displacement, Δr^2 , during the lag-time, Δt , is obtained by the equation,

$$\Delta r^2(\Delta t, t) = (X(t + \Delta t) - X(t))^2 + (Y(t + \Delta t) - Y(t))^2, \Delta t = nt_{\text{intvl}}, \quad (1)$$

where $X(t)$ and $Y(t)$ are the X–Y position of a single molecule at time point t . The time interval t_{intvl} represents the interval for data acquisition and n is a natural number (**Fig. 5a**). The MSDs for each Δt is obtained by averaging the squared displacements, $\Delta r^2(\Delta t, t)$.

3. Fit the MSD- Δt plots with a linear function,

$$\text{MSD}(\Delta t) = 4D\Delta t + a,$$

where D is the diffusion coefficient and a is a constant representing the positioning error of the measurement system. The slopes of the first four data points (i.e., from $n = 1$ to 4 in **Eq. 1**, $\Delta t = 33$ –133 ms) in the MSD plots are used to obtain the SRD (**Fig. 5b**).

4. Create histograms of the SRD. This histogram shows the distribution of diffusion coefficients of target molecules. If the molecules have two types of diffusion states, the SRD histogram becomes broader or has multiple peaks. As an example, an SRD histogram of cAR1 is shown in **Fig. 5c**. This analysis is simple and useful to detect multiple diffusion states, but precise diffusion coefficients cannot be obtained because no appropriate distribution function for fitting the SRD histogram

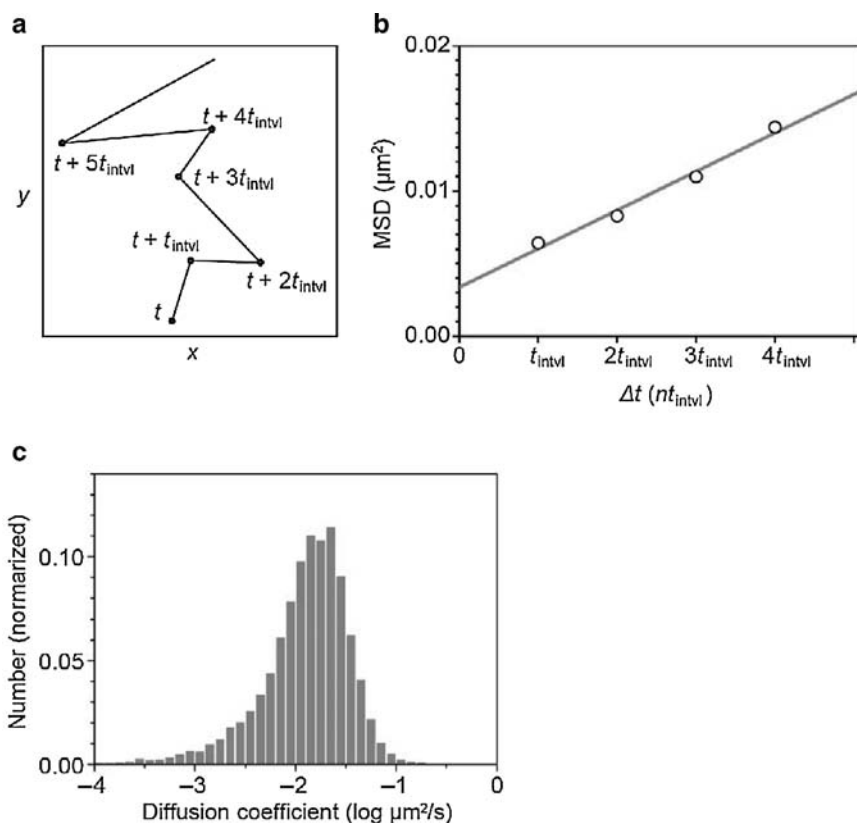


Fig. 5. SRD analysis of cAR1-TMR. (a) Illustration of diffusion analysis. (b) MSD- Δt plot of short trajectory. (c) SRD histogram of cAR1-Halo-TMR.

is available. To obtain precise diffusion coefficients, displacements distribution function (DDF) analysis can be used as described below.

3.7. Diffusion Analysis Based on Distribution Function of Displacements

DDF analysis gives precise diffusion coefficients of target molecules. In this analysis, molecule displacements during a short lag-time are derived from trajectories in order to make the distribution function, which is used to obtain the diffusion coefficients. This method does not require any fitting processes in order to construct the DDF and hence is suitable for precise mobility analysis of target molecules on the membrane. Here we describe the DDF analysis method for cAR1, which has one diffusion state. This analysis can be applied to other molecules that exhibit more complicated behaviors; some molecules exhibit two different types of diffusion states and state transition between them.

1. Construct the data set of displacements with various lag-times (Δt). Displacements (Δr) can be obtained by Eq. 1.

2. Construct the histogram of Δr for a particular lag-time, Δt (**Fig. 6a**). The cumulative histogram of Δr also can be obtained (**Fig. 6b**). The histogram and the cumulative histogram shown are of 200 ms lag-time.
3. Fit the histogram with the equation,

$$P(\Delta r) = \frac{2\Delta r}{MSD_1(\Delta t)} \exp\left(-\frac{\Delta r^2}{MSD_1(\Delta t)}\right)$$

or cumulative histogram with the equation,

$$P(\Delta r) = 1 - \exp\left(-\frac{\Delta r^2}{MSD_1(\Delta t)}\right).$$

Fitting curves were superimposed in **Fig. 6a, b**.

4. Calculate the MSD for the corresponding lag-time, Δt (*see Note 17*). The calculations are done for various lag-times to obtain the set of MSD.
5. Make an MSD versus lag-time, Δt , plot. An MSD- Δt plot of cAR1 is shown in **Fig. 6c**.

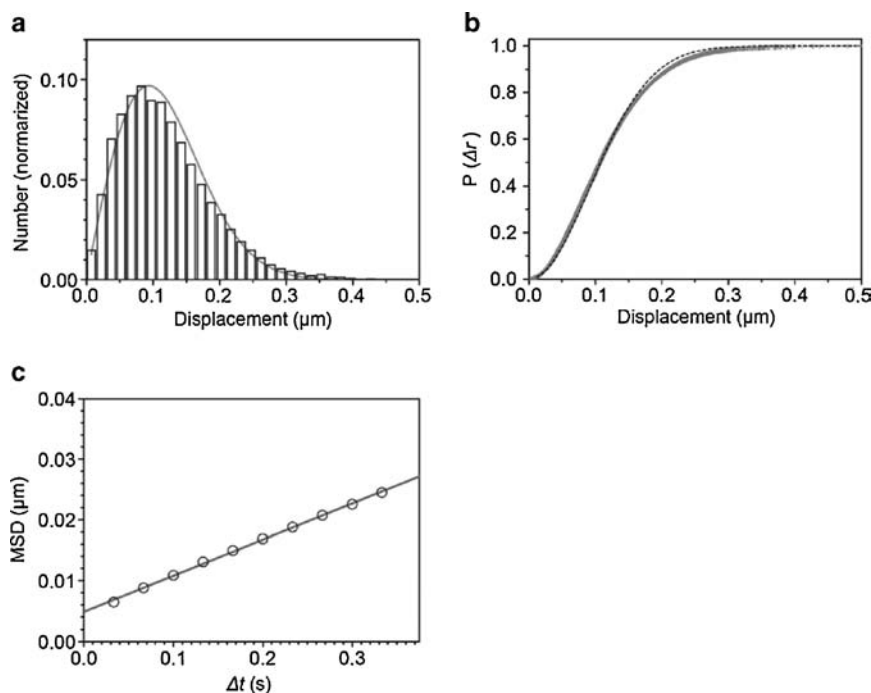


Fig. 6. DDF-based diffusion analysis. **(a)** Displacement histogram of 200 ms lag-time. **(b)** Cumulative displacement histogram of 200 ms lag-time. Both histograms are essentially equivalent. Cumulative histogram is appropriate to apply the least square fitting because cumulative representation is not affected by the bin width of the histogram. **(c)** MSD- Δt plot of DDF-based diffusion analysis. Each data point is obtained by analyzing the corresponding cumulative displacement histogram.

6. Determine the diffusion coefficients by fitting the MSD plots with a linear least square fitting method.

4. Notes

1. There are two types of TIRFM, an objective-type and a prism-type, which depends on the type of experiment. For single-molecule imaging experiments in living cells, we usually use objective-type TIRFM. **Figure 1b** illustrates the typical configuration of an objective-type TIRFM on an inverted microscope. This type of microscopes has free space above the specimen. This configuration can be applied to thicker samples such as living cells. Also, it is relatively easy to combine with other techniques utilized in cell biology, such as microinjection, micromanipulation, and electric recording ([16–18](#)).
2. Objective-type TIRFM requires an objective lens with a very high NA (>1.4), which are commercially available (e.g., NA1.65 Olympus Apo 100× oil High Reso, NA1.45 Olympus PlanApo 100× oil, and NA1.45 Olympus PlanApo 60× oil). These lenses work well for observing living cells. In general, we use NA1.45 objectives for single-molecule observation in living cells.
3. In order to obtain effective total internal reflection illumination, it is important to focus the laser beam precisely on the BFP. The focus can be set by observing the spreading of the laser beam emitted from the objective at a long distance. The spreading should be minimized.
4. Diffusion of the cAMP receptor cAR1 depended on the developmental stages of the *Dictyostelium* cell. cAR1 in the vegetative cells exhibited slower diffusion than that in the developed cells. The SRD histograms of both the vegetative cells and the developed cells are shown in **Fig. 7a**.
5. Wild-type cell lines are chosen as the parental cell line to express the target proteins for single-molecule observations. When cAR1 was expressed in cAR1/cAR3 knockout cell line (RI9), some cells exhibited diffusion in cAR1 mobility that was very similar to vegetative cells (**Fig. 7c**). This may be the result of defects in developmental progression of cAR1/cAR3 knockout cells due to lower expression levels of exogenous cAR1. In cell populations containing a mixture of both the undeveloped and fully developed cells, two diffusion states would be observed in cAR1 mobility. For the same reason, cells lacking molecules essential to developmental progression such as heterotrimeric G proteins should be carefully used

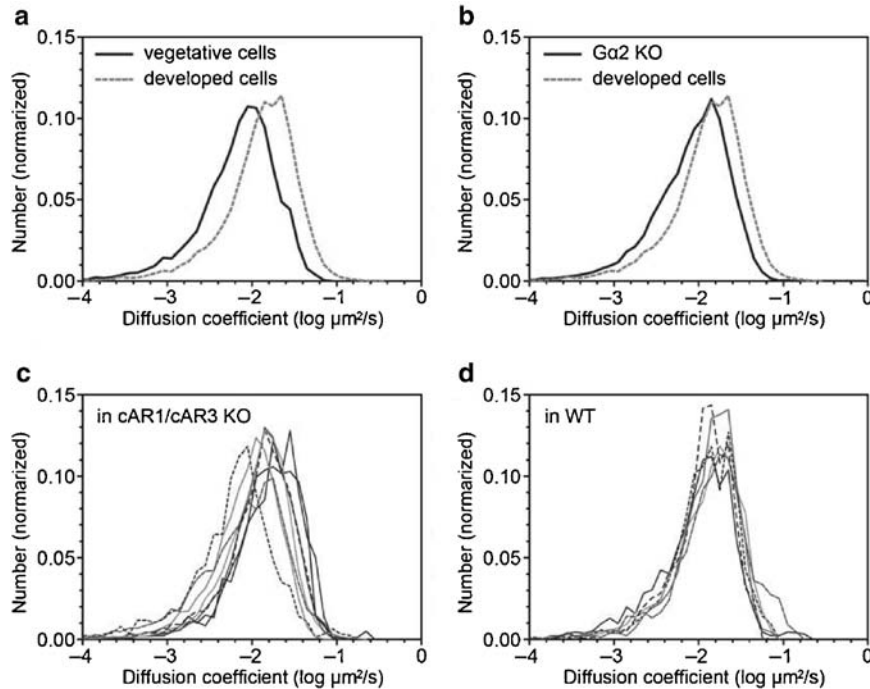


Fig. 7. Effects of developmental progress of cells on cAR1 mobility and phenotypic variability. **(a)** The SRD histogram of both vegetative (*dark line*) and developed (*dashed line*) cells. Vegetative cells exhibited slower cAR1 mobility than developed cells. **(b)** The SRD histogram of cAR1-Halo-TMR expressed in $G\alpha 2$ knockout cells (*dark line*), showing slower mobility similar to that of undeveloped vegetative cells. This is probably due to defects in developmental progress. **(c)** The SRD histogram of cAR1-Halo-TMR expressed in cAR1/cAR3 knockout cells. Each histogram was obtained from individual cells. The SRD histogram shows large heterogeneity among cells, which shows phenotypic variability in cAR1 mobility. Because some cells exhibited a characteristic distribution similar to that of undeveloped vegetative cells, this variability is probably due to defects in development synchronization. **(d)** The SRD histogram of cAR1-Halo-TMR expressed in wild-type cells. In contrast to that of cAR1/cAR3 knockout cells, the SRD histograms of wild-type cells are homogeneous in cell populations.

during the mobility analysis. The SRD histogram of cAR1-Halo that was expressed in $G\alpha 2$ knockout cells is shown in **Fig. 7b**. It is indistinguishable from cAR1-Halo expressed in vegetative wild-type cells. We cannot exclude the possibility that the mobility shift of cAR1 in $G\alpha 2$ knockout cells is due to defects in developmental progression.

6. Expression level of labeled proteins is significant for proper signaling events because excessive expression of target proteins may change the stoichiometry between the target proteins and their upstream (or downstream) molecules in living cells. When the expression levels of target proteins needs to be reduced, we use the expression vector pHK12neo, which lacks the enhancer element in the actin15 promoter region (19).

7. HL5 medium containing DSW distinctly increased the transformation efficiencies by approximately twofold to threefold (20).
8. HaloTag technology has many advantages for fluorescently labeling proteins for single-molecule imaging analysis when compared to other fluorescent proteins such as green fluorescent protein. First, various fluorescent dyes are available for HaloTag. Fluorescent dyes such as TMR are superior in photostability, namely, less blinking and longer photobleaching time (Fig. 4). These properties are critical to obtain precise single-molecule kinetics. Second, labeling efficiency is controllable for HaloTag. A high labeling efficiency is problematic in single-molecule analysis because of potential fluorescent overlap between single molecules.
9. Halo-tagged proteins can be confirmed as functional by examining whether or not the Halo-tagged proteins can rescue phenotypic defects in cells that lack the corresponding gene (9).
10. When we use HaloTag TMR ligand at 50 nM, only a few percentages of the Halo-tagged proteins are labeled with the ligand. For labeling almost all Halo-tagged proteins expressed in cells, use higher concentration of the TMR ligand, e.g., 5 μ M. When the molecules exhibit rapid diffusion, the labeling efficiency may be decreased to avoid possible overlapping between fluorescent spots. Empirically, the densities of the fluorescent spots should be limited to less than ~ 0.1 molecule/ μm^2 for molecules with diffusion coefficients of ~ 0.2 $\mu\text{m}^2/\text{s}$ and less than ~ 0.7 molecule/ μm^2 for molecules with diffusion coefficients of ~ 0.02 $\mu\text{m}^2/\text{s}$.
11. Caffeine is an inhibitor of adenylyl cyclase. Therefore one can achieve the condition of no extracellular cAMP (21). This treatment makes cells sensitive to external cAMP stimulations.
12. The proximity between the cell surface and glass surface is important to achieve TIRFM single-molecule imaging. The agar-overlay method is sometimes suitable to press cells against the glass surface for single-molecule imaging (2, 3, 22). However, this method compromises accessibility to the cells, i.e., cells cannot be stimulated by adding cAMP solution. We used PEI-coated coverslips to achieve both cell adhesion and accessibility. Otherwise, caged cAMP can be used for cAMP stimulations in the agar-overlay method (3).
13. Precise control of the incidence angle is important to generate evanescent fields in a reproducible manner because the intensity and the depth of the evanescent fields depend on the angle. For intensity and penetration depth of the evanescent wave, I_{eva} and d , respectively, we have,

$$I_{\text{cva}} = I_0 \exp[-z/d], \quad d = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2 \theta - n_2^2}} \quad (2)$$

where z is the perpendicular distance from the glass surface and I_0 is the intensity of the evanescent wave at $z = 0$. The penetration depth, d , is the $1/e$ value in **Eq. 2**. λ is the wavelength of the incident light in vacuum, n_1 and n_2 are refractive indices for glass and water, respectively. And θ is the incidence angle measured from the norm (z axis). The theory for TIRFM has been described previously (**11**).

14. TMR has a longer lifetime than YFP. Also, TMR exhibits photobleaching with a single time constant, while YFP exhibits at least two time constants. This suggests that YFP is unstable and frequently exhibits photoblinking.
15. As shown in **Fig. 4d**, lifetime analysis can be applied to other signaling molecules including cAMP and PTEN. Because the lifetime of cAR1-Halo-TMR reflects on the photobleaching times of TMR in living cells, the measurements of cAR1-Halo-TMR provide a good control experiment for other molecules.
16. When the dissociation event is governed by the Poisson process, the probability for remaining on the membrane will decay with time following an exponential function.
17. If those equations are not suitable for analysis of the histogram or the cumulative histogram, use the extended equations,

$$P(\Delta r) = a_1 \frac{2\Delta r}{MSD_1(\Delta t)} \exp\left(-\frac{\Delta r^2}{MSD_1(\Delta t)}\right) + a_2 \frac{2\Delta r}{MSD_2(\Delta t)} \exp\left(-\frac{\Delta r^2}{MSD_2(\Delta t)}\right) + \dots$$

or

$$P(\Delta r) = 1 - \left(a_1 \exp\left(-\frac{\Delta r^2}{MSD_1(\Delta t)}\right) + a_2 \exp\left(-\frac{\Delta r^2}{MSD_2(\Delta t)}\right) + \dots \right),$$

where a_i represents the relative amount of the i th component; that is the summation of a_i equals 1:

$$\sum_{i=1}^N a_i = 1.$$

These extended equations can be used for the analysis of molecules with multiple diffusion states. This model assumes that molecules have multiple populations of different diffusion coefficients. A suitable number of states for data can be determined by a statistical method such as Akaike information criterion (AIC).

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Chapter 29

Imaging B-Cell Receptor Signaling by Single-Molecule Techniques

Pavel Tolar and Tobias Meckel

Summary

B-cell activation initiates antibody responses against pathogens. Recent imaging of B cells in vivo shows that B cells move rapidly through lymphoid tissues to search for antigens captured on the surfaces of antigen-presenting cells. Recognition of antigens by the B-cell antigen receptor (BCR) leads to microclustering of the BCR and the initiation of intracellular signaling that prompts the B cells to stop and form immunological synapses with the antigen-presenting cells. Although the biochemical signaling pathways downstream of the BCR that mediate these events are becoming better characterized, the initial molecular steps in BCR microclustering and activation remain elusive. In part, this is because the dynamics of the cell–cell contact makes the observation of the antigen-induced changes in the BCR technically challenging. Here we review single-molecule imaging techniques that help to provide new information on the molecular behavior of small populations of the BCR as they initiate intracellular signaling in a dynamically moving B cell. The techniques are generally applicable to the study of a broad range of membrane receptors involved in cell–cell contacts.

Key words: Single-molecule imaging, single particle tracking, Receptors, Intracellular signaling, B-cell receptor, Immunological synapse, Planar lipid bilayers

1. Introduction

The dynamic contact of receptors on a moving cell with ligands on other cells warrants specific considerations for the study of activation of intracellular signaling pathways. The small number and the specific distribution of the receptors engaged by membrane ligands lead to a highly localized response with equally local regulation. Often, the fast dynamics of the receptor activation

makes timing of analyses difficult. As a consequence, ensemble experiments that image or biochemically analyze all receptors in a cell as a population, may not capture accurately the initial steps in receptor activation. Here we focus on single-molecule imaging techniques that are fast and sensitive enough to visualize the dynamics of a small number of receptors and provide thus a better view of the initial steps in receptor activation.

Single-molecule imaging is a technique that captures the movement of individual molecules with high spatial and temporal accuracy. There are several advantages of the single-molecule approach. First, because molecules are observed individually, the technique can detect subpopulations of receptors that differ in their behavior. Second, by imaging individual molecules over time, changes in receptor behavior appear as individual events that would otherwise be hidden by averaging a number of asynchronously behaving receptors. And last, the position of individual molecules can be mathematically determined with a better precision than is the limit of optical microscopes, allowing resolution of the receptor movement even in small structures. However, there are also inherent characteristics of the technique that limit the information which can be obtained from recordings or need to be considered during analysis in order to avoid misinterpretations. Both are linked to the photophysical properties of the fluorophores. While photobleaching simply limits the duration over which a single molecule can be observed, photoblinking (i.e., the random switching of the fluorophore between fluorescent and nonfluorescent states), complicates the analysis in various ways (e.g., it can limit the duration of observation or derogate the quantification of single-molecule intensities). However, even given these drawbacks, single-molecule imaging provides an exiting window into the individuality of membrane receptors and complements well traditional assays of receptor function.

Here we describe a protocol for imaging of single BCR molecules on B cells that contact antigen attached to artificial planar lipid bilayers. This experimental setup mimics the contacts of B cells with antigen-presenting cells as it occurs *in vivo*, and allows sensitive and fast imaging of the B cell membrane with total internal reflection (TIR) fluorescence microscopy. We describe how to prepare lipid bilayers decorated with protein ligands, how to label the BCR for single-molecule imaging, how to acquire image streams, and how to analyze the movement of single molecules. The techniques are generally applicable to the study of receptors that mediate adhesion, chemotaxis, and cell activation in cell–cell contacts.

2. Materials

2.1. Preparation of Planar Lipid Bilayers

2.1.1. Preparation of Small Unilamellar Vesicles

1. 8-mL glass vials with Teflon caps (Fisher Scientific, Pittsburgh, PA).
2. 1- and 50 mL Hamilton syringes (Hamilton, Reno, NV).
3. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-[[N(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl], nickel salt (DOGS-NiNTA) (both from Avanti, Alabaster, AL) in chloroform, stored at -20°C .
4. Source of nitrogen or argon gas (*see* [Note 1](#)).
5. Phosphate-buffered saline (PBS), pH 7.4
6. Powerful waterbath sonicator (e.g., Laboratory Supplies Company of New York, Hicksville, NY; or Misonix, Farmingdale, NY).
7. Ultracentrifuge with swinging rotor (Beckman, Fullerton, CA).
8. 0.22-mm polysulfone syringe filters (Whatman, Florham Park, NJ).

2.1.2. Preparation of Planar Lipid Bilayers

1. Glass coverslips 24 × 60 mm, No 1.5 or 1.0 (e.g., VWR, West Chester, PA).
2. Sulfuric acid and 30% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA). Immediately before use mix sulfuric acid and hydrogen peroxide 2:1 (this mixture is sometimes referred to as “Piranha.” Use chemical hood and proceed carefully as the solution heats up during mixing. Alternatively, use a ready-made solution called “Nanostrip” (Cyantek, Fremont, CA).
3. Lab-tek imaging chambers with four or eight wells (Cat.Nos. 155383 and 155411, Nunc Nalgene, Naperville, IL, *see* [Note 2](#)).
4. Ultrapure filtered water.
5. Anhydrous ethanol.
6. Sylgard 164 silicone elastomer (Dow Corning, Midland, MI). Inert, two-part glue, which cures in 30 min and leaves no toxic residuals, making it compatible with live-cell imaging.

2.2. Preparation of Histidine-Tagged Ligands

1. Ni Sepharose (GE Healthcare, Piscataway, NJ).
2. Buffer A: 5 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.
3. Buffer B: 500 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.
4. Gel chromatography column (e.g., Superdex 200, GE Healthcare, Piscataway, NJ).
5. Centrifugal protein concentration units (Millipore, Billerica, MA).
6. Dialysis cassettes (Pierce, Rockford, IL).
7. Hank’s balanced saline solution (HBSS) (Invitrogen, Carlsbad, CA).

2.3. Labeling Receptors by Fluorescent Fab Antibody Fragments

1. Fab preparation kit (Pierce, Rockford, IL).
2. Dialysis cassettes with 3 mL capacity and 10,000 molecular weight cutoff (e.g., Slide-A-Lyzer, Pierce, Rockford, IL).
3. 0.1 M sodium bicarbonate buffer, pH 8.3.
4. Coomassie protein assay reagent (Pierce, Rockford, IL).
5. 1 mg succinimidyl ester of the dye of choice (e.g., Cy3 mono-Reactive Dye Pack, GE Healthcare, Piscataway, NJ).
6. Dimethyl Sulfoxide (DMSO).
7. 1.5 M Hydroxylamine in water, pH 8.5, freshly prepared.
8. Desalt spin columns, 2 mL volume (such as Zeba Spin, Pierce, Rockford, IL).
9. Glycerol.

2.4. Single-Molecule Image Acquisition

2.4.1. Optical Setup

1. Laser (sufficient power for each line to achieve a collimated beam exiting the objective with an intensity of around 3–4 mW). Laser power is best measured at the sample level with a wand power meter (Newport, Irvine, CA).
2. Polychromatic Acousto-Optic Modulator for rapidly switching and choosing laser lines (model number 48062-2.5-.55, Neos Technologies, Melbourne, FL, USA).
3. Inverted microscope with TIR illumination port.
4. TIR capable objective (e.g., Zeiss 100 $\times$, NA 1.45, *see* [Note 3](#)).
5. Dichroic mirrors, bandpass emission filters (Chroma, Rockingham, VT), notch filter (Semrock, Rochester, NY, *see* [Note 4](#)).
6. Back illuminated, frame transfer electron-multiplying charge coupled device (EMCCD) camera (such as Photometrics Cascade II 512, Roper Scientific, Tuscon, AZ). Back illuminated EMCCD cameras capture more than 90% of incoming photons and amplify signals to overcome instrument noise.

2.4.2. Acquisition Software

1. Image acquisition software that can acquire image streams at camera frame rate (e.g., Methamorph, Molecular Devices, Sunnyvale, CA).
2. Scriptable image analysis software (e.g., Matlab, Mathworks, Nattick, MA).

3. Methods

3.1. Preparation of Planar Lipid Bilayers

Here, planar lipid bilayers are prepared by a modification of the original technique described by McConnell et al. ([1](#)). First, small unilamellar vesicles are prepared from phospholipids and then

fused with a clean glass surface. By using phospholipids containing nickel, protein ligands can be attached to the bilayers via histidine tags.

3.1.1. Preparation of Small Unilamellar Vesicles

1. In a glass vial with a teflon cap and using glass Hamilton syringes, mix 22.5 mmol of DOPC with 2.5 mmol of DOGS-NiNTA (ratio 10:1) in chloroform. (This will produce 10 mM lipid solution after reconstitution with 5 mL.).
2. Evaporate chloroform from lipid mixture with a stream of nitrogen or argon (*see Note 1*). Continue to evaporate any residuals of chloroform from the pellet for 2 h in vacuum.
3. Add 5 mL of degassed PBS and fill the vial with nitrogen or argon.
4. Vortex for 30 s. This creates a cloudy solution of multilamellar vesicles.
5. Chill water in a waterbath sonicator with ice and sonicate until the solution is clear, which indicates formation of small unilamellar vesicles.
6. Ultracentrifuge at $65,000 \times g$ for 8 h to remove any larger lipid structures. Collect supernatant.
7. Filter through a 0.22-mm filter and store at 4°C in a tube filled with nitrogen or argon. Due to losses of lipids during centrifugation and filtering, the resulting concentration will be approximately 5 mM. The solution can be stored for up to 6 months.

3.1.2. Preparation of Planar Lipid Bilayers

1. Clean glass coverslips by submerging them into freshly prepared mixture of sulfuric acid and 30% hydrogen peroxide (3:1) for 30 min (*see Note 5*).
2. Rinse coverslips extensively with ultrapure water.
3. Submerge coverslips into ethanol and dry with a stream of nitrogen or argon.
4. Remove glass bottoms from Lab-tek imaging chambers (*see Note 2*).
5. Glue clean coverslips to the bottoms of the chambers with Sylgard 164. Wait 30 min for the glue to cure.
6. Dilute small unilamellar vesicles to 0.1 mM in PBS and apply them to the chambers.
7. Incubate for 10 min at room temperature to allow formation of the bilayers.
8. Wash the bilayers with 10 mL of PBS. Keep the bilayers under water all the time.
9. Bilayers are now ready for binding of protein ligands.

10. To determine the quality of the bilayer, load a fluorescently labeled recombinant protein containing a histidine tag (*see Subheading 3.2*) and image by fluorescence microscopy. The bilayer should appear as a surface with homogeneous fluorescence. To verify mobility of the protein in the bilayer, photobleach a region of $\sim 20 \times 20 \mu\text{m}$ in the bilayer by shrinking the field of view by the field diaphragm and record fluorescence recovery in the region. At 37°C , the fluorescence should recover within 2 min to 80–95% of the original level (*see Note 6*).

3.2. Preparation of Histidine-Tagged Ligands and Their Attachment to Planar Lipid Bilayers

This protocol describes the preparation and attachment of histidine-tagged proteins to the bilayers containing Ni-NTA in the phospholipid headgroups. The main advantage of this technique is that the genetic fusion of the histidine tag with the protein results in a predictable orientation of the protein on the bilayer and maintains protein's natural stoichiometry. Alternatively, proteins can also be attached to lipid bilayers covalently, or by a streptavidin–biotin linkage, but these techniques can result in random orientation and multimerization of the proteins.

The protocol here outlines production of a secreted recombinant protein in mammalian cells and purification via the histidine tag, but other methods of preparation of pure histidine-tagged proteins should work equally well.

1. Clone the protein of interest including the signal sequence and attach a terminal 12 histidine tag using standard molecular biology procedures (*see Note 7*).
2. Prepare stable clones of CHO cells expressing the protein. Growth the CHO cells in 1 L of serum-free media and collect supernatant.
3. Load supernatant on a Ni column at 2 mL/min (*see Note 8*).
4. Wash with two column volumes of buffer A.
5. Elute with a gradient of buffer B into buffer A and collect fractions. Identify fractions containing recombinant protein by absorbance at 280 and pool them together.
6. Verify the presence of a pure protein by SDS page followed by staining with Coomassie blue.
7. Concentrate the protein by spinning in protein concentration units.
8. Purify by gel filtration, pool fractions containing the protein.
9. Dialyze protein into PBS and concentrate to $\sim 1 \text{ mg/mL}$. Freeze aliquots of the protein at -20°C .

To determine conditions for binding of the protein to bilayers resulting in desired protein density in the membrane, we suggest titration of the protein and measuring density using a fluorescently labeled aliquot of the protein.

1. Label an aliquot of the protein with a fluorescent dye (*see Subheading 3.3.2* for protocol).
2. Load the fluorescently labeled protein on bilayers at concentrations ranging from 10 pM to 10 nM. Incubate for 30 min at room temperature and wash away unbound protein with PBS.
3. Take images of the bilayers loaded with various concentrations. At the lowest protein concentration, freely moving single fluorescent molecules should be visible. Count the number of single protein molecules (N) per area (A) and measure the average intensity (I_s) using illumination with laser power L_s . Then measure the fluorescence intensities of the other samples (I_c) using laser power L_c and calculate the concentration C (number of molecules per area) of the protein as:

$$C = \frac{NI_c L_s}{AI_s L_c}$$

4. Use the unlabeled aliquots of the protein at the desired concentration for future experiments.
5. Wash the bilayers with HBSS with 0.5% serum before adding cells to the chambers.

3.3. Fluorescent Labeling of Receptors

Single-molecule imaging has some specific considerations for the fluorescent labeling of cellular proteins. First, fluorescent labels have to be bright and photostable to allow robust detection of single molecules over significant periods. Second, the spatial label density has to be low enough to obtain separate signals for the individual molecules in the images. Third, for the analysis of stoichiometries it is desirable to have each molecule of interest labeled by a single fluorophore, although this is not required for tracking.

3.3.1. Expression of Fluorescent Protein Fusions

Receptors can be labeled genetically by fusion with fluorescent proteins. Doing so ensures that each protein of interest is labeled with exactly one fluorescent tag, an important feature for quantitative single-molecule studies. Both GFP and YFP can be used for single-molecule imaging. All red variants of these proteins are either not monomeric or not photostable enough for serious single-molecule analysis.

Single GFP molecules are more difficult to detect, because they have a lower molecular brightness than YFP. In addition, the 488-nm laser line, which is used to excite GFP, also leads to an excitation of cellular flavins (2), which increases the autofluorescence back-

ground. YFP is brighter and is excited at 514 nm, which barely excites flavins. On the other hand, GFP is more photostable and blinks less than YFP (3). Thus, longer trajectories are generally obtained from single GFPs.

Taken together, the decision whether GFP or YFP should be used mainly depends on the imaging conditions defined by the specimen. If it is problematic to achieve sufficient signal above background, YFP is the better choice as it provides a 50% brighter signal. In all other cases, GFP's higher photostability and, more importantly, lower blinking probability, make it the favorable choice. Regardless of the choice, the expression of the fluorescent constructs has to be low; this is best achieved by selecting stable clones with lowest expression. In most cases, however, it will still be necessary to photobleach cells ahead of recordings, in order to arrive at a single-molecule detection level. Photobleaching must be carried out with careful observation of the cells' viability, as it potentially causes phototoxic effects.

3.3.2. Labeling Receptors by Fluorescent Fab Antibody Fragments

Here we focus on labeling transmembrane receptors by monovalent antibody fragments. The advantage of this approach is that small organic fluorophores can be used that are superior in brightness and photostability to fluorescent proteins. Moreover, lower density of the labeling necessary for resolution of single molecules can easily be achieved by using low concentration of the labeling antibody. The following protocol describes preparation of monovalent Fab antibody fragments from full antibodies and conjugation of the Fab with a succinimidyl-activated small organic fluorophores such as Cy3 and Cy5. Both Cy3 and Cy5, as well as their alternatives (such as AlexaFluor555, AlexaFluor647, DyLight547, DyLight647, Atto647N) produce great results. We suggest starting with Cy3, which is slightly dimmer than Cy5, but far more photostable.

1. Starting with receptor-specific monoclonal antibody, prepare Fab fragments by digestion with immobilized papain and removing the Fc fragments on a protein A column. Follow manufacturer's protocols.
2. Dialyze into bicarbonate buffer and concentrate to 1 mL using a protein concentration unit.
3. Determine antibody concentration by Coomassie protein assay reagent. Follow manufacturer's protocol.
4. Dissolve 1 mg of the succinimidyl ester of the dye in 50 μ L of DMSO and add to the antibody. Mix the solution quickly.
5. Incubate for 1 h at room temperature. Protect the reaction from direct light.
6. Stop the reaction by adding 100 μ L of freshly prepared 1.5 M hydroxylamine; incubate for 30 min.

7. Equilibrate a 5-mL centrifugation gel filtration column with PBS.
8. Load the conjugation reaction onto the column and centrifuge.
9. Collect the flow through that contains the conjugated Fab fragment.
10. Determine the degree of labeling by measuring absorbance of the dye, calculating the dye concentration, and calculating the molar dye/protein ratio. Manufacturers usually specify the molar absorption coefficient of the dye. Concentration C of the dye can then be calculated as:

$$C = \frac{A}{\epsilon}$$

where A is absorbance at the dye's absorbance peak and ϵ is the molar extinction coefficient.

11. Add one volume of glycerol, mix well and store aliquots at -20°C .
12. To label cells, test labeling with 1–100 ng/mL of the antibody for 10 min at room temperature. For subsequent experiments, choose antibody concentration that produces clearly separated fluorescence spots in single-molecule imaging.
13. Wash cells three times with HBSS containing 0.5% serum and add them to the imaging chambers containing the bilayers.

3.4. Single-Molecule Detection and Analysis

Setting up a single-molecule experiment is an iterative process between acquisition and analysis. After the first recording, an initial analysis will yield valuable information, which is used to refine the conditions of the next recording. The main parameters are:

1. The signal intensity of each molecule above background can be optimized by varying the laser intensity and the excitation duration.
2. The density of single-molecule spots needs to fall into a range that allows for the identification and tracking of individual molecules. It can be altered by changing the dye concentration, adjusting the expression level, or applying a certain amount of photobleaching before the recording.
3. The time between each frame should be set up depending on the speed of the molecules. Typical frame rates are between 10 and ~ 200 Hz.

After these parameters have been optimized, the single-molecule signals are recorded into image sequences from which the positions and intensities are determined by an automated process. The positions are then used to create tracks (i.e., the paths the molecules have moved along over time). These tracks provide the basis of all subsequent analysis, which, amongst other

information, yields the diffusion coefficient for the recorded molecular species.

3.4.1. Single-Molecule Image Acquisition

To ensure a fairly constant signal intensity from each fluorophore (i.e., ignoring intensity variations from shot noise, *see* [Note 9](#)), conditions need to be optimized for the following parameters: excitation power (*see* [Note 10](#)), excitation duration (*see* [Note 11](#)), and detector gain (*see* [Note 12](#)). Moreover, the final magnification in the camera image has to be set such that the full width at half maximum (FWHM) of each diffraction limited signal (i.e., single-molecule spot) is represented by at least 1.5 pixels.

1. Start with the laser in the position for epifluorescence illumination. A collimated beam exits the objective.
2. Set the frame rate of the image acquisition to 10–200 Hz. To achieve the fast range of frame rates (i.e., >30 Hz), the active imaging region of the EMCCD camera usually needs to be reduced.
3. Bring a labeled cell into the field of view of the camera and focus on the membrane attached to the coverslip.
4. If the expression level is too high, it is best to photobleach the cell using epifluorescence illumination, since thereby the whole cell will be depleted from an excess of active fluorescent labels. Bleaching with the evanescent field of TIR illumination will only bleach molecules close to the coverslip. Unbleached molecules from regions above the evanescent field would then quickly replete the imaging area causing the area to be too densely populated again.
5. Slowly approach the critical angle, necessary for TIR illumination, until the background intensity drops significantly. If clear and sharp fluorescent signals disappear when focusing to a more distant plane to the coverslip, TIR illumination has been achieved.
6. Select an imaging area, in which, at first, very bright structures should be excluded, as they will make it difficult to see single-molecule signals. They can later be included in the imaging region once the optimal settings have been found. Set the imaging software to automatically scale the dynamic range of the live view (i.e., to set the brightest pixel of the active imaging region to white and the darkest to black).
7. If the density of fluorescent signals is too high, repeat **steps 4** and **5**.
8. Laser power and detector gain have to be set, so that the small spots are just easily distinguishable from the background.
9. Acquire the movement of single molecules by recording multiple image sequences of the same region. Each image sequence

should cover a duration (i.e., the product of frame number and time per frame) long enough to record many single-molecule tracks until their final photobleaching event. While the latter largely depends on the fluorophore being used, 200–500 frames per image sequence are generally sufficient.

3.4.2. Analysis of Single-Molecule Image Sequences

The following section outlines the analysis of the single-molecule image sequences. Raw images are filtered using a spatial band-pass filter (*see* **Note 13**), allowing selection of fluorescence peaks that are higher than the background. The “center of mass” position of the peaks in subsequent frames is then used to connect the peaks into tracks. Next, the position of the peaks in tracks is refined using a 2D Gaussian fit and the resulting coordinates are used to calculate mean square displacements and diffusion. The detection, tracking, and Gaussian fitting of single-molecule signals need to be automated by scriptable software. Many commercial options (e.g., Matlab, IPLab, ImagePro, LabView, Igor Pro) as well as free ones (e.g., ImageJ, Octave) exist to perform this task. Since we use Matlab for our analysis, we found a good basis in Daniel Blair’s and Eric Dufresne’s freely available Matlab adaption of the particle-tracking procedures originally written for IDL by David Grier, John Crocker, and Eric Weeks (<http://physics.georgetown.edu/matlab/>). The following sections, however, give a general description of the required steps, without focusing on any particular software.

Preselection of Peaks

1. The fact that the single-molecule signals have an FWHM equal to or larger than 1.5 pixels (*see* **Subheading 3.4**) allows us to filter the image using a spatial bandpass filter (*see* **Note 13**) so that noise and background variations are removed. The result is a filtered image, which contains the single-molecule signals on a flat and smoothed background (**Fig. 1a, b**).
2. The candidates of single-molecule signals, which exceed a chosen threshold, are selected (**Fig. 1b**). In the remaining description we will refer to these signals as “peaks.”
3. Finally, a “center of mass” algorithm is used to determine the approximate position of each peak.

Gaussian Fits

In order to obtain more precise quantitative information about each peak, its 2D intensity distribution is fitted to a 2D Gaussian (**Eq. 1**). While this step is not necessarily required for subsequent tracking (*see* **Subheading 3.5.4**) each parameter provides useful additional information:

- (a) The center (x_c , y_c) is determined with subpixel resolution (**Fig. 2**).
- (b) The offset (z_0) gives the local background intensity.

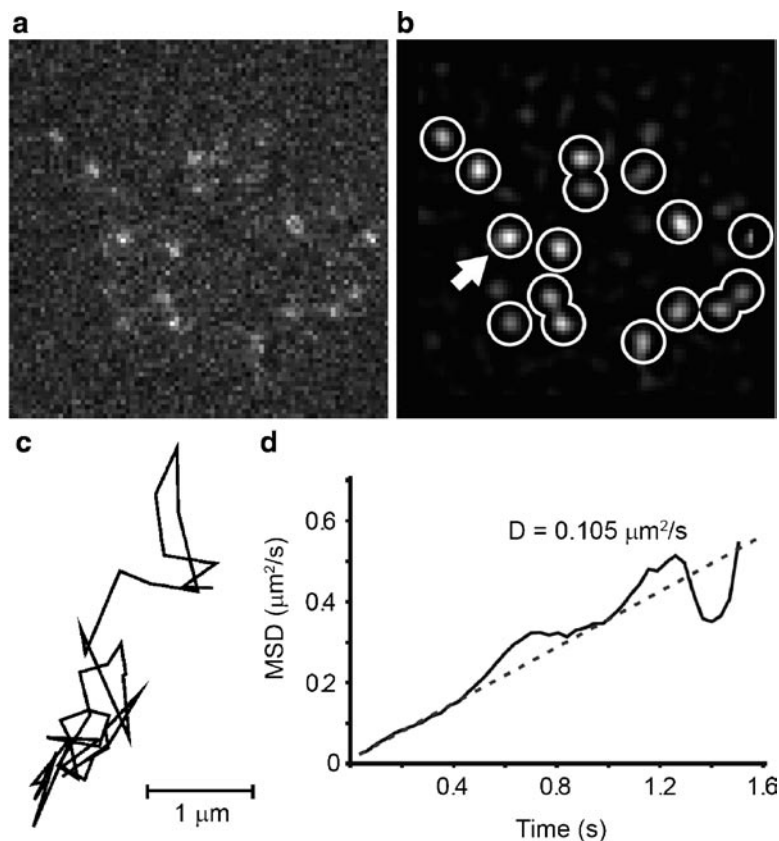


Fig. 1. Example of single-molecule images, tracking, and MSD calculation **(a)**. An image of single BCR molecules labeled with Fab fragments conjugated with Cy3. The image shows a single B cell spread on a bilayer containing ICAM-1. Exposure 35 ms **(b)**. The image after application of a bandpass filter. Circles show spots that were selected for analysis based on a threshold cutoff **(c)**. Trajectory of the molecule indicated by the arrow in B **(d)**. Single-molecule MSD plot for the selected molecule. Dotted line shows a fit to the first ten data points that was used for calculation of the diffusion coefficient D .

- (c) The intensity (I) gives the integrated signal intensity of the peak without contribution of the local background.
- (d) The width (w) gives the FWHM and can identify whether recordings are out of focus or drifted out of focus during image acquisition.

$$f(x, y) = z_0 + 4 \ln 2 \frac{I}{\pi \omega^2} e^{-\left[4 \ln 2 \frac{(x-x_c)^2}{\omega^2}\right]} e^{-\left[4 \ln 2 \frac{(y-y_c)^2}{\omega^2}\right]} \quad (1)$$

1. Choose a single molecule from those detected earlier (*see Subheading 3.4.1*) and crop an area from the unfiltered image around it. The width and height of this area should be approximately five-times the mean FWHM of the peaks.

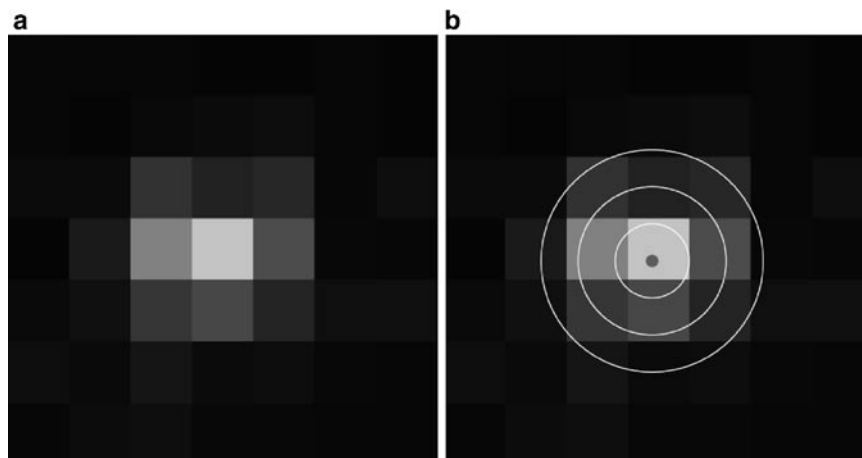


Fig. 2. Visual demonstration of the enhanced positional accuracy achieved by fitting a single molecule with a 2D Gaussian **(a)**. A pixelated diffraction limited spot as it is produced by the fluorescence of a single molecule and captured by the EMCCD camera. The size of a pixel is 150 nm **(b)**. At the signal-to-noise ratio commonly achieved with our single-molecule setup, a 2D Gaussian fit determines the center of the pixelated signal with an accuracy of around 30 nm (*filled gray circle*). The white circles mark the distances corresponding to 1, 2, and 3 times the standard deviation around the center (i.e., the mean) of the Gaussian distribution. They account for 68.3%, 95.4%, and 99.7% of the integrated signal, respectively.

2. Use the center of mass position as an initial guess for the center (x_c, y_c) of the 2D Gaussian fit.
3. Provide the fitting procedure with physically meaningful lower and upper boundaries for each fit parameter, as this will lead to a more robust fitting. The minimum and the maximum of the background (z_0) can only have values between 0 and 2^{16} in a 16-bit image; the maximum of the width (w) can safely be set to the maximum of the area. In the same way, meaningful boundaries for the expected intensity (I) can be given after some initial test recordings have been analyzed. This value varies greatly with camera type and acquisition settings.
4. Based on errors of the estimated parameters and the quality of the fit, peaks can be discarded or the fit can be reattempted with different starting parameters.
5. Store the frame number of the single molecule, the values and errors for all parameters along with the peak number (or some other identifier) in a table, matrix, or a comma or tab delimited text file.

Filter Peaks

If all peaks have been fitted with a 2D Gaussian as described earlier, numerous options for choosing a subselection of peaks exist. The rationale behind such a subselection can be manifold, and while the following list is not exhaustive, it exemplifies the general idea:

- (a) By allowing only peaks to survive the selection process, whose fitting errors fall below a certain threshold, a dataset can be cleaned of peaks that did not fit well to the shape of typical diffraction limited signals. Hence, these signals might not have been caused by single fluorophores.
- (b) Selecting only peaks whose intensity values fall within a certain range. Likewise excludes fits of structures other than those of single fluorophores.
- (c) Excluding peaks with a width beyond a certain value, limits the focal range within peaks are allowed to reside in.

Tracking

From the previous fitting steps we obtain positions for all single-molecule signals in all consecutive frames of the original image sequence (**Fig. 1c**). In order to connect these positions to trajectories and thereby follow the mobility of each particle in time, we need to define a maximum movement ($r_{\max}$) which a molecule is allowed to make within a single time step (i.e., the time between two consecutive frames). A trajectory is then built as follows:

1. A circle with radius $r_{\max}$ is drawn around each peak in frame n .
2. If a peak within one of these circles is found in frame $n + 1$, a connection is made to the corresponding peak (i.e., the peak in the center of that circle) in frame n .
3. If more than one peak is found within one circle in frame $n + 1$, the trajectory is terminated in frame n .
4. Since an incorrectly chosen $r_{\max}$ will yield wrong results for both the trajectories themselves as well as calculations based on them (such as diffusion coefficients), the value for this input parameter must be chosen with great care (4). If $r_{\max}$ is chosen too small, the final dataset will not contain any long movements, leading to an underestimation of the true diffusion coefficient. If $r_{\max}$ is chosen too large, the probability for connecting two different molecules as being part of one track increases. Obviously, $r_{\max}$ should be smaller than the mean distance between the peaks in the same frame. The optimal value for $r_{\max}$ is found when small changes in $r_{\max}$ do not cause a change in the calculated diffusion coefficient. This requires, as mentioned earlier, an iterative analysis of some small part of the recorded data.

3.4.3. Analysis of Single-Molecule Tracks

The analysis of single-molecule data is a field of ongoing development. We therefore only provide a relatively simplified view on a basic approach to obtain the diffusion coefficient from the analysis of single-molecule tracks. In principle, each track can be analyzed individually, which yields a diffusion coefficient for each tracked molecule. However, due to the stochastic nature of the

data, it is advisable to include the information of a large number (e.g., 1,000) of molecules, before the calculation of a diffusion coefficient. All recorded tracks of the same molecular species will be treated as a single pool of information about this molecule. Since the movement of a single molecule is a stochastic process, the distribution of the displacements is the same, regardless if displacements are part of one or many separate tracks. Thereby, useful information can be obtained even from recordings with limited track length, which is usually the case for recordings of fluorescent proteins. To obtain a diffusion coefficient from a single track, the mean square displacements need to be analyzed as a function of time. To keep things simple, we will use a track with three steps:

1. The first square displacement (SD) is calculated between positions 1 and 2 of the track with the coordinates (x_1, y_1) and (x_2, y_2) , respectively. Pythagoras' formula then yields the value of the first square displacement:
2.
$$SD = (x_2 - x_1)^2 + (y_2 - y_1)^2$$
3. The procedure is repeated for all displacements, which are separated by a single timelag. A timelag is the time between each frame of the image sequence. We therefore calculate the SD between positions (1,2), (2,3), and (3,4).
4. In the next step, we calculate the SD for all positions separated by two timelags. The corresponding positions are (1,3) and (2,4) and do the same for the positions separated by three timelags, which for our example is (1,4).
5. The arithmetic means of all SD with identical timelags are now calculated and plotted against the respective timelag. This yields a mean square displacement (MSD) plot, which describes the mobility of the molecule in dependence of the observation time (**Fig. 1d**).
6. Assuming that the movement of our example molecule follows the model of Brownian motion, we can fit the plot with the function $MSD = 4Dt$ (with t = timelag) to obtain the diffusion coefficient (D) for the molecule (**Fig. 1d**).
7. For the combined analysis of multiple tracks we simply repeat **steps 1–3** for each track, and apply **step 4** to the data obtained from all tracks. The MSD plot now represents the combined behavior of all recorded molecules. Consequently a fit of the function mentioned in **step 5** will yield the diffusion coefficient of the whole population of recorded molecules. If the fit significantly deviates from the data, the movement cannot be described by the model for Brownian motion. In that case other models for molecular motion may apply (**5**).

4. Notes

1. We use argon or nitrogen tanks. To obtain dust-free stream of the gas, it is best to insert a 0.45- μm syringe filter before ending the line with a clean Pasteur pipette.
2. Any chambers can be used, but we found that the chambers with the indicated catalog numbers have coverslip bottoms that are the easiest to separate.
3. All major microscope manufacturers have now dedicated TIR objectives. A considerable issue with objective-type single-molecule TIR is that the high-power laser beam travels through the objective to the specimen and then reflects back. Scattering and autofluorescence by the objective's optical system can lead to a significant background signal in the emission path. We found Zeiss 100 $\times$ NA 1.45 to have exceptionally low fluorescence background even when using high laser power.
4. Although the dichroic mirror and the emission filter should theoretically prevent laser light from entering the camera and increasing background, we found that many emission filters were not sufficient to block the high-power laser light completely. Adding a notch filter that specifically blocks the laser wavelength with high efficiency usually resolved the issue.
5. We found convenient to use glass slide containers (Fisher Scientific). The containers will hold the coverslips during this step as well during subsequent washes.
6. Various imperfections of the bilayers are common. These may include immobile fluorescent spots or small areas of glass not covered by the bilayer. These local imperfections usually do not affect the quality of the bilayer in other regions of the coverslips. However, an overall reduction in the mobile fraction of the fluorescent protein usually means poor quality of the bilayer, most often caused by improperly cleaned coverslips.
7. We found that using 12 histidines, as opposed to six, leads to a more stable attachment to bilayers (6).
8. The subsequent steps are best performed using an HPLC system. Alternatively, manual operation of the column and loading with a peristaltic pump is also possible.
9. Due to the "particle" nature of photons, it is unlikely that each pixel on the EMCCD camera receives the exact same number of photons per time. Consequently, the value of each pixel, which contributes to a single-molecule signal, will vary over time.
10. Beyond a certain excitation intensity, the signal intensity of a single molecule does not increase. Beyond this "saturation intensity" only the probability for bleaching increases.

11. If the excitation duration and the frame rate of the acquisition cannot be controlled independently (i.e., via an AOM, *see Subheading 2.4.1*), the excitation duration is equivalent to the time between two frames. The slower the frame rate, the longer the excitation duration, the brighter the single-molecule signals.
12. While signal-to-noise ratio (SNR) is improved by increasing the EM gain of an EMCCD camera, there is a limit to this improvement. Depending on the signal intensity, the maximal SNR is reached well below the maximal EM-gain setting. The optimal EM-gain setting (i.e., the setting beyond which no further SNR improvement is achieved) has to be determined for every imaging situation. Since the signal intensities of single-molecule recordings fall into a narrow range, a single setting for the EM gain can be found for all recordings.
13. A spatial bandpass filter smoothes the noise and removes background variations from the image. The cutoffs of the bandpass are chosen, so that signals smaller (i.e., noise) and larger (i.e., background variations) than the signals of interest are suppressed. Background variations are removed by subtracting from the image a lowpassed version of the image and noise is overcome by convolving the image with a Gaussian kernel.

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Chapter 30

Light Microscopy to Image and Quantify Cell Movement

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Summary

For decades, *Dictyostelium discoideum* has been an efficacious and attractive model system for the study of cell motility, primarily because cells become highly motile during the transition from growth phase to aggregation competence and because the haploid genome is readily amenable to mutation. These crawling amoebae, as well as other motile cells such as polymorphonuclear neutrophils (PMNs), extend pseudopodia, retract pseudopodia, and translocate across a substratum even in the absence of chemoattractant. This phenomenon, referred to as basic motile behavior, has been investigated in *Dictyostelium* through analysis of cytoskeletal mutants. Likewise, many chemotactic signal transduction pathways and networks have been inferred from studies of *Dictyostelium* mutants. However, before concluding from mutational analyses that a particular molecule or protein plays a role in chemotaxis, it is imperative to first precisely define its contribution, if any, to basic motile behavior. Here, we describe two-dimensional and three-dimensional technologies that can be coupled with 2D and 3D Dynamic Image Analysis System (2D and 3D-DIAS) software for the analysis of cell motility, shape changes, pseudopod formation, and localization of tagged molecules during basic motile behavior. In addition, we describe a method to analyze the 3D trajectories of microspheres attached to the surface of crawling *Dictyostelium* cells. We include information on microscopy, image acquisition techniques, and computer hardware that could be reproduced in a typical laboratory setting for motion analysis using 2D and 3D-DIAS software. Finally, we highlight features available in DIAS that have proven insightful in identifying defects in basic motile behavior exhibited by various cytoskeletal and putative signal transduction mutants.

Key words: Cell motility, DIAS, Confocal microscopy, 2D motion analysis, 3D motion analysis, Basic motile behavior

1. Introduction

Although cells are three dimensional, we perceive them as two dimensional because we usually view them through traditional compound microscopes from on top or through inverted microscopes from underneath. In addition, although behavior

can be complex especially when it involves the extension and retraction of pseudopods, we tend to track cells over time as dots in a two-dimensional plane. Thus the perception of cellular translocation along a surface is usually oversimplified and in some cases inaccurate, especially when they are the basis for models of cellular translocation or interpreting defective behaviors in mutants (*1*).

Here we describe methods for reconstructing cells and analyzing their motion in 2D and 3D that are based on the collection of outlines of live, migrating cells at one plane for 2D analysis and in the z-axis in a short time frame for 3D analysis. The method involves the use of the software programs 2D-DIAS and 3D-DIAS (*2–10*). The 2D system is in place in over 75 universities and institutes, and the 3D system remains in a laboratory format that can be accessed by a visit to the W.M. Keck Dynamic Image Analysis Facility at the University of Iowa. Given its applicability, the general formats for running these systems should serve as models for the development of 2D and 3D systems by other scientists. Here we describe the capabilities and methods for application of these systems to single-cell motility studies.

The object of the 2D system is to obtain a sequence of outlines of the cell perimeter, pseudopod, and nucleus that are mathematical models and can thus be used to quantitate changes in shape and motility over time. The objective of the 3D system is to generate a sequence of 3D reconstructions in time that are mathematical models and thus allow the quantitation of dynamic shape and motility parameters in 3D. This must be done with a microscope system that provides the resolution of detail necessary to answer the questions posed with minimal damage on the normal shape and motility of the cell. Any high-contrast microscope system can be used for 2D studies, but differential interference contrast microscopy provides the optimum imaging platform for 3D analysis of amoeboid cells. For 2D analysis, a cell crawling on a glass, quartz, or plastic surface is continuously recorded at one plane usually providing the most information on the cell body and pseudopods. For 3D analysis, a cell crawling on a glass, quartz, or plastic surface is optically sectioned through a depth predetermined that encompasses all z-axis dynamics. For a cell 15–20 μm long, such as a human polymorphonuclear leukocyte or *Dictyostelium* amoebae, 60 optical sections collected in a 2-s period through 10 μm is more than sufficient. This procedure is normally repeated every 5-s. The images are collected and introduced into the DIAS programs in a digital movie format. The sequence of 2D images is automatically or manually outlined and the outlines converted into beta-spline representations that are amenable to motion analysis. The software system delivers a sequence of

outlines, “difference pictures,” motility parameters based on the track of the computed centroid of the image (i.e., center of area), shape parameter based on contour, and digital movies of translocating cells. The stacks of optical sections for each 3D reconstruction are also outlines and the outlines converted to beta-spline models. These are then connected in the z-axis to generate the encapsulating 3D surface at each time point. These mathematical models can then be used to generate sequences of 3D images, motility, and shape parameters and computer movies. For both the 2D and 3D reconstructions, pseudopods or cell extensions can be encapsulated and independently reconstructed and motion analyzed. The same is true for the nucleus and uropod.

2. Materials

2.1. Culturing Cells

1. HL-5 growth media ([http://www.dictybase.org](http://www.dictybase.org;); (11)).
2. *Dictyostelium discoideum* amoebae are grown to the low-log phase (2×10^6 cells/mL HL-5) in suspension cultures shaking at 125 rpm (see [Note 1](#)).

2.2. Development of *Dictyostelium discoideum* on Filter Pads

1. *Dictyostelium* BSS: 20 mM KCl, 2.5 mM MgCl₂, 20 mM KH₂PO₄, 5 mM Na₂HPO₄ (pH 6.4), 0.34 mM streptomycin sulfate, filter sterilize (12–14).
2. Black filter paper (<http://www.thomassci.com/catalog/product/2067>) and 47 mM Millipore support filters AP1004700 (<http://www.millipore.com>).
3. Humidity chamber at 22°C.

2.3. Two-Dimensional Imaging for Analysis of Basic Motile Behavior

1. Microscope equipped with a long working distance condenser sufficient to accommodate perfusion chambers and 10× to 40× objectives.
2. Sykes-Moore perfusion chamber with inlet and outlet ports (12, 13), (Bellco Glass, Inc., Vineland, NJ) or custom-made microfluidic device.
3. NE-1000 Multiphase Programmable Pumps (New Era Pump Systems, Farmingdale, NY) (15) or peristaltic pump to replace the volume of BSS in the chamber every 15 s (see [Note 2](#)).
4. CCD camera connected to an ADV55 analog-to-digital converter (Canopus, Co., LTD, <http://www.canopus.com>). Alternatively, one can use a Firewire or other digital camera (see [Note 3](#)).

5. Firewire, IEEE 1394 compatible image acquisition software such as iStopMotion, iMovie or QuickTime Pro®, version 7 or later for Macintosh, or Adobe Premiere® and QuickTime Pro, version 7 or later with Windows XP or Windows Vista operating systems.
6. 2-D DIAS® software (Soll Technologies, Inc., Iowa City, IA), QuickTime Pro version 7 or later (*see* [Note 4](#)) and a G3, G4, G5 or Intel-based Macintosh computer with OS X version 10.3, 10.4, or 10.5, 128 MB Ram or more and 20 MB hard disk space.

**2.4. Imaging for 3-D
DIAS Analysis of the
Cell Body, Pseudopods,
Filopodia, and Nucleus
During Basic Motile
Behavior**

1. Short working distance perfusion chamber suitable for DIC optics such as the Dvorak-Stotler chamber or the Bioptechs chamber (<http://www.bioptechs.com>).
2. NE-1000 Multiphase Programmable Pump (New Era Pump Systems, Farmingdale, NY) or other means to continuously perfuse BSS with a minimum of peristaltic oscillations (*see* [Note 2](#)).
3. DIC optics on a microscope with N.A. = 1.3–1.4 condenser and 60 or 63×, high N.A. objective.
4. Motorized stage, external motor with controller, or a piezo objective collar along with the means to distinguish up from down slices in a z-series (*see* [Note 5](#)).
5. Camera and video acquisition software (*see* [Subheading 2.3, items 4 and 5](#)).
6. Computer capable of grabbing and storing images at 29.97 frames per second (fps) (*see* [Subheading 2.3, item 6](#)).
7. 3-D DIAS software; for filopodia, DIAS 4.0 on a Windows XP or Mac OSX computer ([16](#)).

**2.5. Confocal Imaging
for 3D-DIAS
Reconstruction
of Internal Cell
Architecture
in Crawling Cells**

1. *Dictyostelium* cells labeled or transformed with a fluorescent tag.
2. Perfusion chamber such as the Sykes-Moore chamber (*see* [Subheading 2.3, item 2](#)). This chamber works well in confocal or multiphoton microscopy when used with quartz coverslips (SPI Supplies Division of Structure Probe Inc., West Chester PA; <http://www.2spi.com>) for improved stability during perfusion and the NE-1000 Multiphase Programmable Pump (New Era Pump Systems, Farmingdale, NY) (*see* [Subheading 2.3, item 3](#)).
3. Software to export images from the native acquisition format to a series of TIFF images (*see* [Note 6](#)).
4. 3-D DIAS software ([5–10](#)).

2.6. Application of 3D-DIAS to Quantitation of Trajectories of Microspheres Attached to the Cell Surface

1. Concanavalin A, from *Canavalia ensiformis* (jack bean) type VI.
2. Polybead polystyrene microspheres. 465 μm diameter $\pm$ 0.11 μm (Polysciences Inc.).
3. BSS (*see* **Subheading 2.2, item 1**).
4. VibraCell Sonicator (Sonics and Materials Inc., Newton, CT <http://www.sonics.biz>).
5. Dvorak-Stotler or other live cell imaging chamber suitable for narrow depth of field, DIC optics.
6. 3-D DIAS software (**5–10**).

3. Methods

3.1. 2D-DIAS Analysis of Basic Motile Behavior

3.1.1. Sample Preparation for Analysis of Basic Motile Behavior

1. For development on filter pads: Pellet 5.5×10^7 growth phase cells, wash three times in BSS, resuspend in 1 mL BSS, and disperse on a 47-mM-diameter black filter paper supported by two Millipore prefilters (*see* **Subheading 2.2, item 2**) saturated with BSS. Incubate in a humidified chamber at 22°C.
2. At the onset of aggregation (approximately 6–7 h on filter pads; (**17**)), wash cells from the filter pads and dilute to a concentration of 3×10^4 cells/mL for motion analysis.
3. *Dictyostelium* strains that are defective in cAMP signaling can be stimulated by exogenous pulsing. Harvest cells in the low log phase of growth, wash three times in BSS, resuspend in 20 mL BSS at a cell density of 5×10^6 /mL, and maintain in suspension on a rotary shaker at 200 rpm. Pulse cells every 6 min for six subsequent hour (**18**) by programming the NE-1000 Multiphase Programmable Pumps (New Era Pump Systems, Farmingdale, NY) to deliver 100 μL of 10 μM cAMP from a 10-cc syringe (**15, 18**).
4. Inoculate 1.1 mL of cell suspension into the Sykes-Moore perfusion chamber. Allow cells to adhere to the coverslip for 5 min at room temperature. During that time, cells should elongate and resume translocation.
5. Connect the chamber to the pump or other perfusion system and place it on the microscope stage. Take care to purge air from all tubing and ports.

3.1.2. Image Capture

Acquire movies with the equipment described (*see* **Note 7**). Movies should be saved in QuickTime format (.mov) for importing into DIAS (*see* **Note 6**).

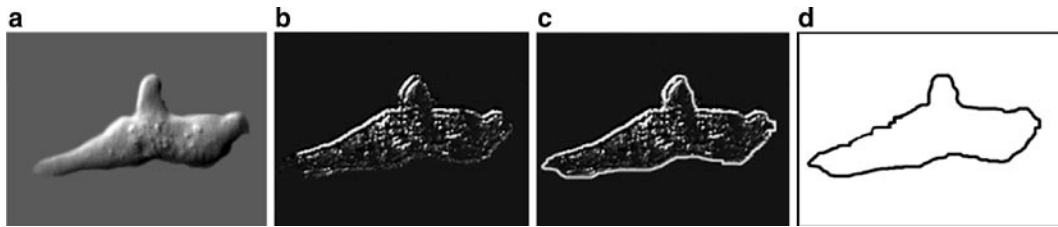


Fig. 1. Outlining by complexity. In this case, the original image (a) of a *D. discoideum* amoeba was obtained with differential interference contrast microscopy. (b) Initial complexity outlining. (c) Final outline. (d) Outer edge with internal architecture subtracted. Dilation of 3 and erosion of 2 was used.

3.1.3. Outlining with DIAS

For automatic outlining, the edge may be detected directly or inferred from detail implying an edge (see Fig. 1). Edges can be automatically detected by thresholding, using either a direct cutoff method or a gradient method, or a complexity method, all described mathematically in a previous, more detailed analysis of the methods (3–10). Selection of the best method must be based on empirical tests. DIAS provides two unique manual outlining options if editing is warranted.

3.1.4. DIAS Path Files

Once the cells of interest in a movie have been outlined, DIAS subtracts all background information, leaving only the perimeter outlines and centroid positions for each cell over all frames in a path file. Subsequent shape analyses and computations (see Subheading 3.1.5 and 3.1.6) are performed on the path file.

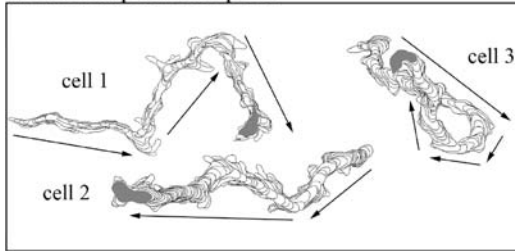
3.1.5. Shape Analysis

1. Smooth contours for accurate measures of cell shapes are achieved in 2D-DIAS by replacing the series of lines that comprise the original outline with mathematically precise beta-splines (3–10).
2. Cell shape changes can be quantified from the beta-spline replacement images by several methods, including stacked perimeter plots (see Fig. 2a) and difference pictures (see Fig. 2b–d). In a difference picture, the outline of the cell at frame n is overlaid with the outline at frame $n-x$ where x is the user-specified frame increment. Expansion zones are regions in the later image that do not overlap the earlier image (filled areas in Fig. 2b–d) and retraction zones are regions in the earlier image that do not overlap the later image (hatched areas in Fig. 2b–d). Both perimeter plots and difference pictures can be saved as QuickTime movies for presentation.

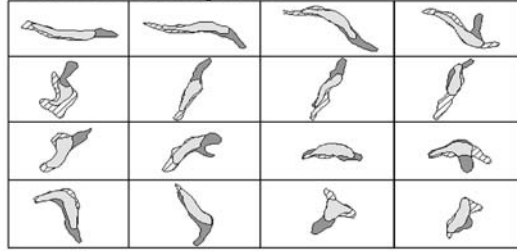
3.1.6. Windowing to Compute Relative Flow

Quantitative measurements of localized expansion and retraction zones (relative flow) can be achieved by windowing the area of interest in a difference picture (see Fig. 3). This measurement has proven essential in identifying defects in pseudopod expansion, pseudopod retraction, and pseudopod area that would otherwise have gone undetected in numerous *Dictyostelium* mutants (19–23).

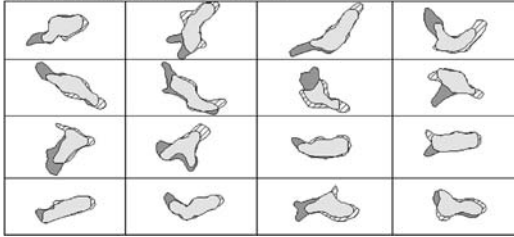
a Stacked perimeter plots



b Cell 1 difference pictures



c Cell 2 difference pictures



d Cell 3 difference pictures

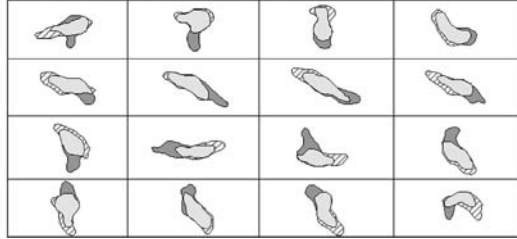


Fig. 2. 2D-DIAS generated stacked perimeter plots (**a**) and difference pictures (**b–d**) of three wild-type *Dictyostelium* cells migrating in buffer. Perimeter outlines in (**a**) are stacked at 8-s intervals for a total of 10 min. The last image in the series is filled with gray. Arrows show direction of travel and turning. Difference pictures in (**b–d**) are overlaid at 8-s intervals. Expansion zones are coded dark gray, retraction zones are hatched, and common zones are light gray. These images are used to evaluate behaviors such as turning and pseudopod dynamics.

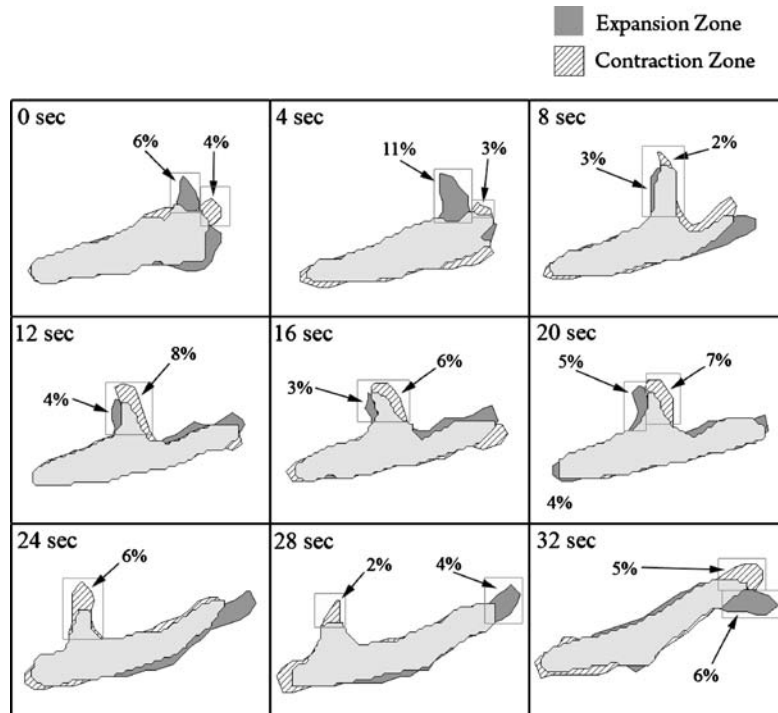


Fig. 3. Windowing of expansion zones (dark gray) and retraction zones (hatched) in a difference picture gives quantitative data on pseudopod dynamics. The user selects the “relative flow” feature from the DIAS pull down menu and then windows the region of interest. DIAS computes the area of expansion and retraction within the window and returns the values as per cent of the total area of the cell.

3.1.7. Computing Parameters

1. The standard DIAS 3.4.2 menu allows the user to select up to 40 parameters and/or take advantage of the built-in advanced RPN calculator to enter user-designed functions as desired. Instantaneous velocity, direction of travel, and direction change are computed from the centroid position (7, 8, 10, 11, 20; see Fig. 4a). The cell centroid can be computed by either the perimeter or area method. The latter method is recommended for *Dictyostelium* and any other cell type that forms lateral pseudopods (4).
2. The DIAS generated database file (see Fig. 4a) contains numeric data for each frame for each selected parameter, while the summary file (see Fig. 4b) contains the average values of selected parameters. The built-in graphics package provides additional functionality including smoothing, finding minima and maxima, linear and quadratic fits, and Fourier transforms. These and other functions can be applied to a user-specified time interval or the entire range of data. Database files and summary files can also be exported into spreadsheet software such as Sigma Plot® or Microsoft Excel® for other statistical and graphics options.

a

Cell	Total time (min)	Total path length (μm)	Net path length (μm)	Dir. pers.	Up. dir.	CI	Speed (μm/m)	Dir. (deg)	Dir. change (deg)	Accel. (μm/m ²)	Pers. (μm ² /m ²)	Centr. X (pixel)	Centr. Y (pixel)	Axis tilt (deg)	Max. length (μm)	Mean length (μm)	Max. width (μm)	Centr. Area (μm ²)	Per. (μm)	Rnd. (%)	Pred. vol. (μm ³)	Pred. surf. area (μm ²)	Rad. length (μm)	Rad. Mean conc. (deg)	Mean flow (deg)	Pos. flow (%)	Neg. flow (%)		
1	9.93	60.8	20.3	0.33	0.27	0.20	4.4	126.3	53.8	-0.58	0.44	166.3	58.3	122.9	16.4	5.3	8.5	6.0	83.5	41.5	64.6	422.0	217.3	5.7	28.9	507.6	147.6	6.6	6.6
2	9.93	93.9	17.9	0.19	-0.05	-0.18	6.9	173.3	57.4	-0.21	0.54	57.0	68.7	126.3	14.8	3.2	5.7	3.4	47.5	36.4	45.6	297.7	120.6	4.6	40.2	520.6	163.0	16.0	14.9
3	4.13	60.5	27.0	0.45	-0.32	-0.31	12.1	232.6	46.1	3.04	0.68	55.4	138.0	60.0	12.8	3.4	6.3	4.5	42.8	33.6	50.2	220.0	109.7	4.2	34.8	529.4	169.4	21.8	19.6
4	2.73	40.4	15.7	0.39	-0.36	0.13	11.2	292.6	38.4	6.62	1.35	481.7	155.7	113.1	13.4	3.8	6.5	3.6	38.1	37.9	51.2	218.7	147.9	4.9	37.1	537.8	177.8	19.2	15.6
5	9.93	91.2	15.1	0.17	0.12	0.12	6.9	47.3	53.9	0.71	0.61	267.2	92.0	64.5	11.9	3.3	5.6	3.7	39.1	30.9	53.4	202.6	100.6	3.9	35.8	506.5	146.5	13.9	12.8
6	3.27	73.2	42.1	0.58	-0.28	0.50	19.7	323.0	27.1	-2.27	3.03	586.6	155.6	103.3	19.4	4.2	8.0	4.7	80.3	49.9	42.8	730.0	204.6	6.1	38.7	624.8	272.0	26.3	23.4
7	9.93	72.1	23.6	0.33	-0.01	-0.33	6.1	180.6	40.7	0.63	0.73	136.0	165.4	78.6	11.7	4.3	6.7	5.0	49.5	30.9	71.3	275.8	129.8	4.2	25.2	454.1	94.1	9.2	8.9
8	9.93	73.0	17.7	0.24	-0.14	-0.19	5.6	208.4	55.5	-1.00	0.57	323.3	218.1	75.7	15.0	4.2	6.7	4.3	62.9	38.8	52.9	389.2	161.8	5.0	36.6	546.0	186.0	10.7	10.0
9	9.93	175.2	87.8	0.50	0.00	0.50	16.3	354.0	26.2	-2.36	2.58	540.7	177.7	98.9	17.4	3.0	5.5	3.5	50.9	42.0	38.7	349.5	128.5	5.2	42.7	559.8	199.8	18.3	17.3
10	9.93	41.4	7.8	0.19	-0.12	-0.14	3.3	206.8	54.2	0.31	0.38	188.4	317.7	29.7	10.9	5.2	7.3	5.9	56.6	29.1	85.3	310.4	152.0	4.3	16.9	413.2	33.2	5.4	5.4
11	9.93	54.9	14.9	0.27	-0.20	0.19	4.2	320.7	48.1	0.11	0.43	345.4	330.2	139.5	12.5	4.2	6.7	4.6	51.8	32.0	65.4	280.9	134.3	4.4	29.3	468.2	108.2	7.8	7.8
12	9.93	96.2	22.1	0.23	-0.13	0.19	7.3	343.5	48.0	-0.80	0.68	450.0	413.9	102.5	12.2	3.4	5.4	3.8	40.2	30.0	57.4	148.6	103.3	4.0	34.4	477.6	117.6	12.6	12.1
13	9.93	138.9	31.9	0.23	0.19	-0.13	10.9	138.6	46.8	-2.26	1.06	260.2	438.7	103.3	13.0	4.3	6.9	4.8	54.0	33.6	63.2	269.2	140.6	4.5	30.2	489.0	129.0	16.0	15.6
14	9.20	86.6	14.7	0.17	0.11	-0.13	7.8	147.5	38.9	-1.25	0.78	230.9	172.6	112.5	12.9	3.0	5.6	3.3	37.8	33.1	44.0	190.3	96.1	4.1	39.8	518.4	158.4	15.1	14.6
15	6.53	48.6	11.2	0.23	-0.04	-0.23	5.9	172.1	44.5	-1.27	0.49	492.0	182.6	111.3	10.3	4.1	6.3	4.6	41.6	27.5	71.4	189.4	109.6	3.8	25.5	454.8	94.8	11.8	10.8
16	3.27	88.7	64.3	0.72	-0.23	-0.69	26.0	195.8	19.2	-32.42	5.87	521.8	232.9	32.6	21.9	3.3	5.6	4.0	71.4	50.7	35.2	690.6	179.0	6.4	44.7	579.1	219.1	23.5	23.8
17	9.93	115.7	41.0	0.35	0.01	-0.35	9.2	164.6	40.5	-0.27	1.23	366.9	80.8	87.2	16.9	4.6	7.5	5.0	76.9	44.6	49.5	507.7	196.8	5.6	37.9	562.6	232.6	11.1	11.0
18	9.93	99.1	59.6	0.60	-0.59	0.12	8.1	280.7	42.2	-0.77	0.70	147.7	210.0	107.3	17.2	4.1	6.6	4.1	69.4	42.9	48.4	301.1	176.9	5.4	38.9	604.0	244.0	11.5	11.1
19	9.93	127.2	95.6	0.74	-0.13	-0.72	11.4	191.6	27.5	-0.89	1.37	243.4	215.8	60.8	19.1	5.0	8.2	5.9	94.9	49.6	49.4	844.5	242.4	6.1	37.8	615.1	255.1	11.0	10.8
20	9.93	97.0	38.3	0.39	0.32	-0.22	8.5	124.1	38.7	1.72	1.44	151.9	129.3	71.7	13.2	3.9	6.5	4.4	51.6	34.2	56.8	261.3	133.0	4.5	34.1	529.5	169.5	12.3	11.7
21	5.60	73.3	40.7	0.56	-0.56	-0.01	12.0	262.2	29.0	-0.16	1.30	256.5	241.8	94.3	14.5	3.8	6.2	3.9	54.5	36.6	52.2	219.6	139.3	4.7	37.5	544.7	184.7	14.0	14.0
22	7.40	90.1	25.0	0.28	-0.27	-0.08	9.9	263.8	45.1	-0.01	1.15	181.7	260.9	55.0	17.6	4.2	7.5	5.0	72.9	45.6	46.0	484.8	186.0	5.6	38.0	631.7	271.7	12.7	12.2
23	7.73	91.7	22.9	0.25	-0.23	0.09	10.9	307.4	41.1	1.13	0.99	304.7	294.9	125.3	18.1	5.0	9.3	6.2	88.2	48.8	48.3	657.0	226.6	5.8	35.4	653.5	293.5	15.3	13.2
24	9.93	95.3	51.4	0.54	-0.54	-0.02	8.3	265.4	38.0	0.51	0.94	321.6	370.1	84.9	16.0	4.6	7.7	4.7	72.9	42.2	52.5	371.5	187.3	5.3	35.8	590.8	230.8	9.2	8.7
25	5.67	69.3	47.1	0.68	0.09	-0.67	11.0	171.5	30.4	0.12	1.27	267.0	345.6	90.8	17.6	4.3	6.6	4.0	75.0	43.7	50.0	445.3	191.0	5.6	38.4	567.9	207.9	12.0	12.1
26	9.40	45.5	19.6	0.43	0.17	-0.40	3.9	150.7	43.7	-0.18	0.29	392.7	355.6	135.7	16.1	7.0	10.3	7.6	112.8	43.9	74.0	814.7	298.9	6.2	22.8	526.8	166.8	4.3	4.4
27	9.93	99.0	49.4	0.39	0.17	0.36	8.7	15.8	31.8	1.31	0.72	172.8	406.5	129.1	15.1	4.2	6.7	4.8	63.2	38.0	56.1	382.4	162.1	5.0	34.8	548.5	188.5	10.4	10.1
28	9.60	130.4	13.9	0.10	-0.04	0.09	13.2	348.8	21.7	-0.36	2.20	402.2	348.8	122.4	14.6	4.5	6.9	4.9	65.1	36.9	60.8	357.1	168.0	5.0	34.4	516.4	156.4	13.8	12.8
29	5.13	65.6	40.5	0.62	-0.12	-0.61	10.9	185.0	33.3	-2.91	1.88	553.2	417.2	39.0	16.0	4.3	7.2	4.8	68.2	40.3	53.5	487.1	174.8	5.2	35.9	571.3	206.7	13.7	13.7
30	3.33	44.1	34.5	0.78	-0.02	0.78	12.2	359.4	20.4	-0.29	1.94	92.1	73.2	89.2	18.5	4.6	7.5	4.7	85.3	46.0	51.3	807.3	217.4	5.9	37.8	594.5	252.0	12.0	12.2
31	9.9	114.2	29.0	0.25	0.1	0.2	9.3	26.2	50.0	-0.5	0.7	218.8	68.4	105.9	16.9	3.9	7.5	4.8	64.8	46.3	39.2	413.9	164.8	5.3	39.4	671.9	311.9	14.5	13.9
32	6.1	75.0	32.2	0.43	-0.4	-0.1	10.4	270.9	42.0	-0.3	1.3	454.2	189.2	90.8	17.5	3.8	8.9	5.2	65.6	50.0	33.9	426.7	166.5	5.5	40.2	701.6	345.4	14.5	14.1
33	11.94	119.4	50.8	0.43	0.4	0.1	11.0	93.2	32.4	0.3	1.1	101.2	108.9	98.1	15.1	3.6	6.5	4.1	53.7	39.8	43.7	310.1	137.0	4.8	39.9	600.2	240.2	15.4	14.6
34	4.4	37.6	27.4	0.73	0.4	0.6	7.5	28.2	26.2	3.0	0.9	323.9	289.7	45.4	16.6	3.3	6.6	3.2	55.1	43.4	37.4	386.0	139.3	5.1	42.8	612.9	258.2	11.8	10.6
35	8.9	125.3	39.5	0.32	-0.1	0.3	12.3	342.8	34.5	-0.4	1.4	306.7	360.3	91.5	17.1	3.4	7.3	3.9	58.0	47.1	33.8	342.5	146.6	5.4	41.5	658.2	298.2	16.4	15.5
36	3.3	47.1	16.7	0.35	-0.2	-0.3	11.3	219.8	37.8	3.5	1.1	391.1	261.5	104.9	16.8	4.2	8.3	5.2	70.1	46.1	42.5	493.6	179.1	5.5	37.3	635.6	275.6	16.3	16.3

b

SYNOPSIS			
Total time (min)	Mean ± Std. Dev.	Centr. X (pixel)	Mean ± Std. Dev.
7.9 ± 2.6		312.6 ± 155.7	5.1 ± 0.7
Total path length (μm)	85.2 ± 32.5	Centr. Y (pixel)	35.5 ± 5.9
Net path length (μm)	33.3 ± 10.9	Axis tilt (deg)	559.9 ± 67.2
Dir. pers.	-0.4 ± 0.2	Max. length (μm)	200.2 ± 67.7
Upward dir.	0.8 ± 0.3	Mean width (μm)	13.5 ± 4.6
CI	-0.04 ± 0.4	Max. width (μm)	7.1 ± 1.1
Speed (μm/min)	9.8 ± 4.4	Centr. width (μm)	12.8 ± 4.2
Direction (deg)	209.1 ± 98.5	Area (μm ²)	63.5 ± 16.8
Dir. change (deg)	39 ± 10.3	Per. (μm)	40.1 ± 6.7
Accel. (μm/min ²)	-0.8 ± 5.7	Rnd. (%)	52 ± 11.8
Pers. (μm/min ²)	1.3 ± 0.1	Pred. vol. (μm ³)	404.5 ± 184.4

Fig. 4. (a) Example of tabular data generated by 2D-DIAS. (b) The summary file with averages and standard deviations.

3.2. 3D-DIAS for the 3D Reconstruction of Live Cells During Basic Motile Behavior

3.2.1. Sample Preparation

3.2.2. Optical Sectioning, Outlining, and Reconstructing Live Cells Using 3D-DIAS

Harvest *Dictyostelium* amoebae at the onset of aggregation (*see* Subheading 3.1.1, steps 1–3) and inoculate 300 μL into the Dvorak-Stotler chamber according to methods described elsewhere in detail (24). Allow cells to adhere for 5 min. before starting perfusion.

1. The greatest challenge in optical sectioning of live cells is synchronization of three essential elements: a camera with a resolution of at least 640×480 , a high-speed motor with reproducible positional accuracy, and the transfer of the z-series information in “real time” (30 fps) to a hard drive with sufficient space to store individual movies that are 2–3 GBs or larger. Commercially available integrated systems generally require some customized adaptations and may be prohibitively expensive.
2. Optical sectioning of live cells is performed using high resolution, narrow depth of field, DIC optics as described in Subheading 2.4, item 3. The motor is programmed to move 10–20 μm for *Dictyostelium*, in 2–3 s.
3. With these settings, 60 optical sections can be obtained in 2–3 s. The process is repeated every 4–5 s for 10 min or longer.
4. Images are written directly onto a computer hard drive using the equipment described in Subheading 2.2, items 4 and 5 at a rate of 30 fps.
5. 3D-DIAS uses a pixel complexity algorithm with user-defined threshold and smoothing to automatically outline the in-focus perimeter of each optical section. Furthermore, the pixel complexity outlining process allows the user to use different settings for different z-slices. The pseudopod/cell body boundary can be manually delineated. In some cases, intracellular organelles can be automatically outlined, but manual editing is generally required. Detailed methods for outlining and reconstructing the cell, pseudopod, and nucleus are described in detail elsewhere (6–10). A representative series of ten optical sections with outlines of the cell body, pseudopods and the nucleus at indicated increments is presented in Fig. 5. Outlining of multiple features in a given frame is accomplished through the use of trace slots, described elsewhere in detail (16, 25).

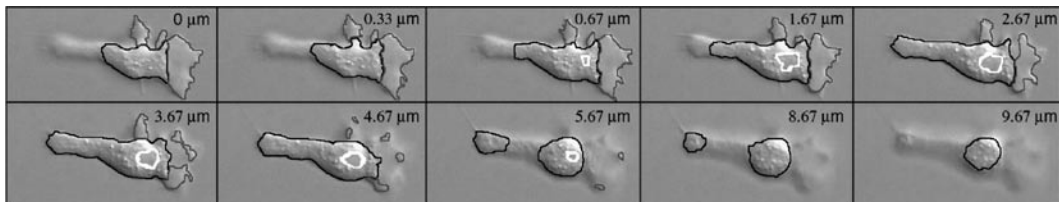


Fig. 5. Outlining optical sections from a DIC image in 3D-DIAS. The in-focus edges of the cell body (black), pseudopods (gray), and nucleus (white) are outlined in different trace slots. Examples are presented at the indicated heights beginning with the substratum (0 μm).

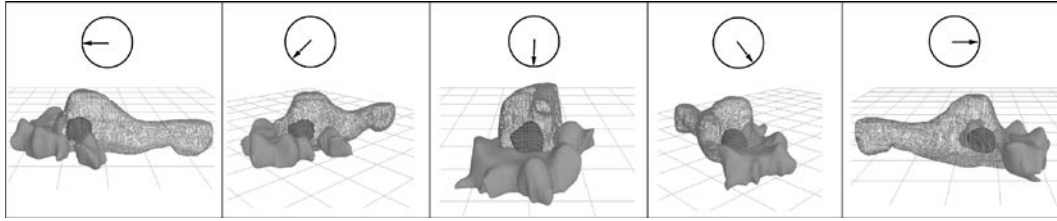


Fig. 6. The cell body, pseudopods, and nucleus are reconstructed from the outlines and can be viewed at any angle and rotation. The cell presented here is at a 30° angle and rotated through a 180° arc as indicated by the arrows above each image.

6. A composite 3D reconstruction is generated from combined or separated trace slots and can be rotated and viewed at any angle (see Fig. 6). The 3D reconstruction can also be saved and viewed as a QuickTime movie.

3.2.3. Motility and Dynamic Morphology Parameters Computed by 3D-DIAS

1. DIAS generates a database file of 3D parameters based on the position of the cell centroid and the 3D contours of the cell body that can be plotted and analyzed as described in Subheading 3.1.3 for 2D database files (see Fig. 7a, b).
2. 2D parameters can also be measured from a 2D projection of the 3D path file.

3.3. 3D-DIAS for the Reconstruction of Filopodia

3.3.1. Sample Preparation for the 3D Reconstruction of Filopodia

Harvest *Dictyostelium* amoebae at the onset of aggregation as described in Subheading 3.2.1, and inoculate into the Dvorak-Stotler chamber or other live cell imaging chamber that allows narrow depth of field imaging with high numerical aperture, high resolution DIC optics (16, 24).

3.3.2. Optical Sectioning, Outlining, and Reconstructing Filopodia in 3D

1. Optical sectioning is performed as described in Subheading 3.2.2. Due to the complex 3D trajectory of filopodia and their narrow diameters (<0.1 μm), video compression is avoided during acquisition and processing of the movie to JDIAS format. Java-based DIAS 4.0 (16) is used for reconstruction because of the software's ability to use uncompressed video, and very high-resolution OpenGL graphics for visualization.
2. Autotracing features available in JDIAS 4.0 are avoided due to the physical nature of filopodia; these structures are more accurately traced manually (see Fig. 8a). Software enhancement techniques are used to further resolve filopodia. In order to accurately render the fine structure of filopodia, the facet limit of each reconstructed portion of the cell within the software was extended to 200,000 per object in JDIAS 4.0 through the use of complex independent thread execution. Additional resolution up to 500,000 facets among the total reconstructed object was obtained by using the OpenGL

a

Cell	3D Speed ($\mu\text{m}/\text{min}$)	3D Dir. pers.	Dir. change (deg)	Volume (μm^3)	Surface area (μm^2)	3D Rnd. (%)	Mean Rad. Length (μm)	Rad. Dev. (%)	Height (μm)	Bulk Height (μm)	Overhang (%)	2D Speed ($\mu\text{m}/\text{min}$)	2D area (μm^2)	2D Rnd. (%)
1	20.0	115.1	0.0	761.1	776.4	37.4	6.8	36.7	8.1	7.6	15.6	13.4	169.8	48.1
2	20.0	115.1	1726.1	954.4	960.3	34.1	7.1	35.4	9.6	9.4	13.4	13.4	164.7	45.7
3	9.9	104.6	157.8	910.0	946.7	33.2	7.1	35.0	9.9	9.1	20.3	5.6	161.5	49.9
4	19.7	115.5	163.5	672.0	728.2	36.4	6.7	37.6	8.4	7.8	17.1	13.5	146.8	51.6
5	20.6	105.4	150.7	826.4	801.6	38.7	7.2	38.5	8.9	8.6	15.0	9.7	158.0	52.0
6	16.0	98.5	104.4	858.7	892.0	34.3	7.2	38.1	9.6	9.1	12.3	14.2	154.7	54.4
7	8.4	-35.7	2012.5	865.7	927.9	32.6	7.3	37.3	11.4	10.4	32.8	3.2	152.2	54.7
8	24.9	104.4	2101.9	649.6	691.2	38.0	6.8	40.4	9.6	8.6	23.8	7.9	145.8	56.1
9	13.2	65.1	589.8	690.2	757.5	35.2	7.0	39.3	13.4	9.4	32.6	13.6	140.3	56.4
10	13.9	99.6	517.0	774.5	898.9	30.6	7.2	39.7	13.7	12.2	35.7	2.7	140.2	48.6
11	7.0	57.4	632.7	995.1	993.2	33.8	7.4	36.4	13.4	12.4	36.9	5.9	142.5	52.3
12	12.3	111.8	816.0	1055.9	990.7	36.0	7.6	35.8	14.9	12.4	23.4	16.4	146.9	56.4
13	23.9	107.4	65.1	854.8	765.3	42.9	6.9	34.7	11.6	10.4	17.6	9.6	141.1	62.1
14	11.5	-157.7	1422.9	874.0	843.1	38.0	7.2	37.6	12.7	10.1	22.1	10.6	144.0	50.4
15	12.7	155.3	705.1	944.1	900.1	37.2	7.4	38.2	11.4	9.9	22.4	12.2	149.9	46.0
16	3.9	35.0	1803.7	1068.9	1039.9	33.9	7.6	35.0	13.4	10.4	26.8	4.6	158.2	51.4
17	40.0	125.8	1361.2	437.1	440.6	50.3	6.1	40.1	6.1	5.6	2.2	16.6	130.8	56.5
18	17.3	-84.1	2251.9	775.0	758.2	39.5	7.1	40.4	9.6	8.6	9.4	8.4	151.3	47.2
19	8.6	150.1	1887.5	899.5	875.2	36.9	7.4	38.9	9.4	8.4	10.7	8.7	159.9	48.2
20	15.3	141.8	123.9	988.0	943.1	36.3	7.4	37.3	10.6	9.6	13.1	16.3	160.0	52.4
21	14.0	148.5	99.7	1048.6	1024.6	34.0	7.6	39.4	11.1	10.1	17.3	10.0	159.7	50.3
22	30.7	65.8	1239.8	888.5	912.0	34.3	7.2	38.6	9.9	9.1	21.1	36.2	150.0	50.0
23	18.0	-6.0	1077.0	1056.7	1107.7	30.5	7.5	37.9	10.4	10.1	14.2	14.6	156.7	46.8
24	33.9	6.0	180.4	651.7	630.1	43.8	6.9	38.8	6.6	5.6	10.2	13.4	158.1	48.3
25	15.9	-13.0	285.9	724.8	765.9	36.4	6.9	38.2	7.8	6.1	12.5	6.9	159.9	48.5
26	16.4	-26.3	199.7	851.0	900.0	33.5	6.9	35.2	8.9	8.1	26.0	8.2	174.0	46.9
27	4.7	-48.6	333.8	816.3	909.2	31.7	6.9	35.3	10.6	8.6	32.7	2.3	164.5	46.7
28	22.3	139.8	2574.9	902.0	944.8	33.0	7.1	35.7	11.9	10.1	22.5	15.0	165.1	45.4
29	28.3	115.5	363.2	593.1	621.6	40.7	6.8	38.7	6.3	6.1	13.2	12.3	161.3	45.1
30	30.6	116.6	15.4	809.1	801.3	37.9	7.4	38.6	8.4	7.6	19.6	24.8	180.8	47.4
31	36.0	118.6	31.1	834.2	859.4	35.2	7.8	40.3	8.1	8.1	15.1	31.5	179.4	48.6
32	39.1	102.1	247.5	721.8	771.4	35.8	7.5	43.5	8.9	8.9	33.7	44.8	169.2	48.8
33	26.3	81.9	303.2	762.7	847.7	32.9	7.2	41.6	9.6	9.4	25.8	20.5	159.0	50.1
34	24.8	65.3	249.0	598.0	602.6	43.0	6.8	39.4	8.9	6.8	13.5	4.3	158.5	52.9
35	8.5	-60.9	1893.9	676.9	632.3	45.3	6.8	41.2	7.8	7.6	20.3	3.9	164.9	55.9
36	14.5	47.8	1630.8	840.1	791.8	40.1	7.1	39.1	9.6	9.4	23.6	7.7	170.5	57.6
37	12.9	-162.9	2240.2	882.1	884.8	35.6	7.1	37.2	10.6	10.4	25.0	12.7	168.0	53.6
38	10.7	-172.6	146.3	879.8	858.1	37.2	7.2	38.8	10.1	9.9	21.7	11.6	162.5	50.6
39	35.4	117.6	1047.3	559.4	559.2	45.0	6.7	42.3	7.1	6.6	18.4	11.2	162.0	48.9
40	16.6	-29.1	2199.5	679.2	701.6	38.9	7.0	42.1	9.4	9.1	38.0	4.0	171.6	44.9
41	12.8	-142.3	1699.1	770.1	816.5	35.1	7.4	42.5	11.1	10.9	46.7	7.1	171.5	40.2
42	21.9	-35.6	1601.6	785.4	825.8	35.2	7.1	39.3	12.4	12.2	37.2	16.7	162.6	53.0
43	27.1	-2.7	492.8	808.1	797.1	38.2	6.6	33.1	11.1	10.1	22.4	30.6	163.4	54.0
44	30.4	37.1	597.5	510.1	501.6	48.3	5.9	34.6	7.1	6.3	10.4	23.3	147.9	58.8
45	13.9	-36.3	1101.3	583.8	575.0	45.0	6.3	34.5	7.3	6.1	9.4	13.5	156.4	49.4
46	11.5	-43.1	102.1	688.5	718.0	38.1	6.6	37.0	7.6	7.6	25.8	10.1	166.1	45.4

b

SYNOPSIS			
Parameter	Mean $\pm$ Std. dev.	Parameter	Mean $\pm$ Std. dev.
3D Speed ($\mu\text{m}/\text{min}$)	19.1 $\pm$ 9.3	Radial dev. (%)	38.2 $\pm$ 2.4
3D Dir. persistence	41.6 $\pm$ 91.4	Height (μm)	9.9 $\pm$ 2.1
Dir. change (deg)	881.4 $\pm$ 791.7	Bulk Height (μm)	8.9 $\pm$ 1.8
Volume (μm^3)	799.5 $\pm$ 148.8	Overhang (%)	21.3 $\pm$ 9.3
3D Roundness (%)	810.7 $\pm$ 146.0	2D Speed ($\mu\text{m}/\text{min}$)	13.1 $\pm$ 8.9
Surface area (μm^2)	37.4 $\pm$ 4.5	2D Area (μm^2)	158.3 $\pm$ 10.9
Mean rad. length (μm)	7.1 $\pm$ 0.4	2D Roundness (%)	50.6 $\pm$ 4.4

Fig. 7. 3D-DIAS generates a data file that can be viewed in tabular (a) as well as summary (b) form. Parameters can also be computed from a 2D projected path.

(Silicon Graphics) 3D graphics libraries to communicate with an NVIDIA GeForce 8800 GTS graphics card for visual representation. DIAS controls the lighting, color, texture, size, and view angle of the image through the software's built-in OpenGL processing station (see Fig. 8b, c).

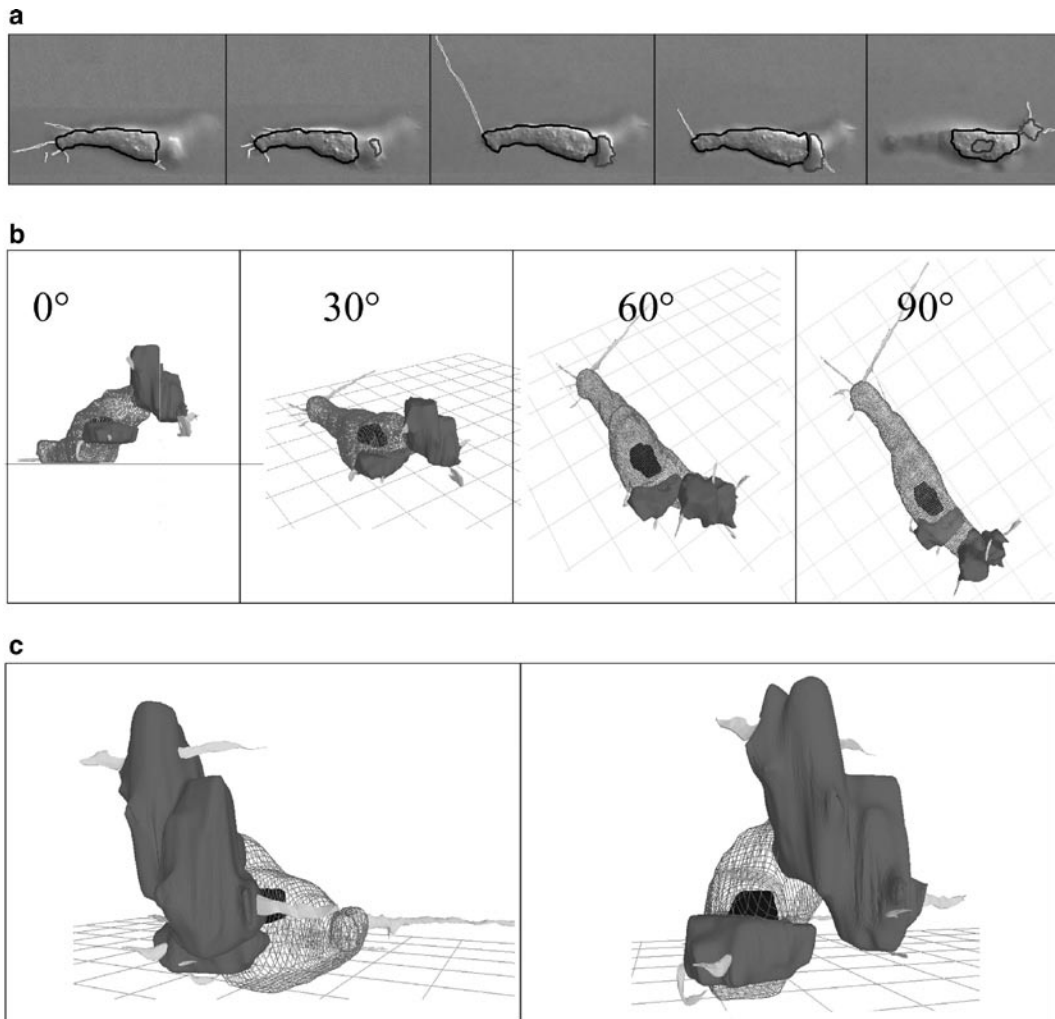


Fig. 8. (a) Outlining filopodia in optical sections (b) 3D reconstructions of cell body, pseudopods, nucleus, and filopodia at indicated angles and (c) A magnified view of filopodia at two different rotational angles.

3.4. 3D Reconstruction of Live Cells from Confocal Images for Localization of Tagged Molecules During Basic Motile Behavior

3.4.1. Sample Preparation

3.4.2. Image Acquisition

1. Cells expressing a fluorescently marked compound, protein, or molecule are inoculated into a Sykes-Moore perfusion chamber with quartz coverslips (SPI supplies, West Chester, PA).
2. The chamber is connected to a perfusion system that minimizes oscillations.
3. Transmitted and fluorescent images of cells in buffer are collected using the appropriate laser line and a 60x planapochromat water immersion objective (N.A. 1.2).

A rapid scan rate (166–500 lines per second) is generally required for crawling amoebae. For best results, the signal should be sufficiently bright to permit collection of a minimum of ten slices of a 512×512 image at 4–5 s intervals.

- Using the native acquisition software, the image series must be batch converted to a TIFF sequence and imported into QuickTime Pro 7. The QuickTime movie format is DIAS compatible.

3.4.3. Outlining Images Using Trace Slots

- The user can take advantage of trace slots (16, 25) to outline and quantify different intensity levels of a given label. For the cell reconstructed and viewed at different angles in Fig. 9, actin is labeled with the ABD–GFP fusion protein probe (26). In this figure, the cell body, represented as a transparent faceted reconstruction, is outlined in trace slot 1 while intensely labeled actin, seen in anterior and lateral pseudopods, is in a separate trace slot. A lower intensity of labeled actin, automatically outlined in trace slot 3, can be seen to localize primarily to the uropod.

3.5. 3D-DIAS for Analysis of Surface Microsphere Movements

3.5.1. Preparation of Sample

- Dilute 2 mg/mL of Concanavalin A in BSS.
- Incubate 40 μL /mL of polystyrene microspheres in the Concanavalin A and BSS solution for 30 min at 4°C.
- Centrifuge at 3,600 rpm for 30 min, pour off supernatant, and resuspend the pellet in 10 mL of BSS. Repeat.
- Sonicate with Vibracell sonicator (Sonics and Materials Inc.) at 15% amplitude for 20 s.
- Harvest cells as described in Subheading 3.1.1, steps 1 and 2. Mix 80 μL of polystyrene microspheres/Concanavalin A solution with 4 mL of cells at 1×10^5 cells/mL in BSS. Immediately inoculate the mixture into the Dvorak-Stotler chamber.

3.5.2. Optical Sectioning, Image Acquisition, Outlining, 3D Reconstruction with 3D DIAS, and Analysis of Surface Microsphere Movements

Because a cell is a three-dimensional entity that changes shape in the x, y, and z axes as it crawls, it is necessary to perform 3D reconstructions in order to get accurate information on the behavior of particles attached to the cell surface. To measure the trajectories and compute 3D parameters of microspheres attached to the surface of *Dictyostelium* amoebae, optical sectioning, image acquisition, and outlining using trace slots are performed as described

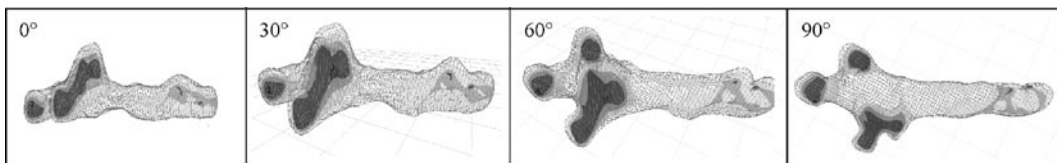


Fig. 9. Laser scanning confocal microscope acquired z-series of a *Dictyostelium* cell expressing a GFP-tagged actin-binding domain (23) in BSS. A laser scanning rate of 250 lines per second was used at 1- μm increments over 10 μm . The user can define separate threshold values so that DIAS automatically outlines different intensity levels in different trace slots. Trace slot 1 contains outlines of the cell body, while trace slots 2 and 3 contain outlines of midrange and high pixel intensity, respectively.

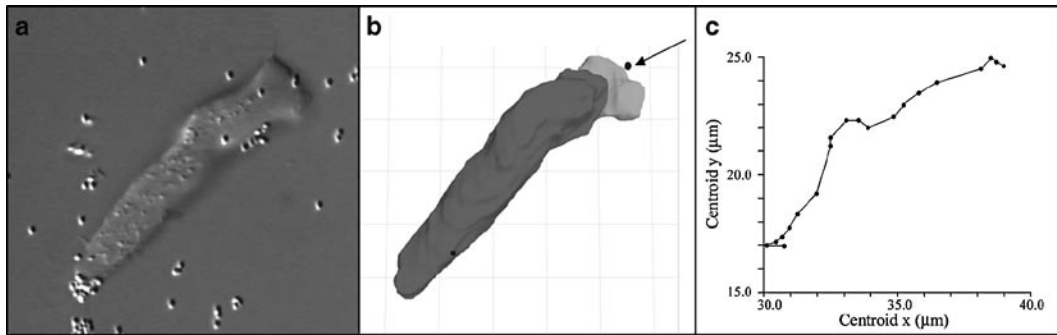


Fig. 10. DIC, substrate level image of a *D. discoideum* with (a) polystyrene microspheres surrounding and settled onto the cell. (b) The OpenGL 3-D reconstructed image of the *D. discoideum* amoeba with the cell body, pseudopodia, and each polystyrene microsphere placed in a separate JDIAS 4.0 trace slot to aid in calculations. (c) The movement of the polystyrene microsphere attached to the pseudopod plotted for 2 min

elsewhere in detail (see Subheading 3.2.2 and (6, 22)). A typical substrate level image of a cell with attached microspheres is presented in Fig. 10a. In this case, a bead at the tip of the anterior pseudopod was chosen for analysis. A 3D reconstruction of the cell body (*gray*), anterior pseudopod (*light gray*), and the microsphere (*black*) are presented in Fig. 10b. The centroid track of the microsphere over time is presented in Fig. 10c.

4. Notes

1. Many *Dictyostelium* mutants do not divide in suspension cultures; therefore, in order to attain sufficient cell number, these strains are grown to the monolayer stage in a 150-mm Petri dish in 20 mL HL-5 supplemented with antibiotics.
2. It is essential to continuously refresh the microenvironment during the recording so that cAMP and phosphodiesterase released by the cells will be rapidly removed and not affect basic motile behavior.
3. Using inexpensive analog-to-digital converters allows the user to employ analog CCD cameras to acquire digital images with a very small investment in new technology. Firewire cameras are slower and generally more expensive. High-speed digital cameras capable of capturing video at 30 fps may also be cost prohibitive.
4. QuickTime Pro supports dozens of compressions and video codecs; these provide a balance between compression, quality, and file size. While MPEG standard H264 offers the best quality to file size ratio, it also requires the most processing

power to decode. 2D and 3D DIAS are capable of handling any one of these formats, but for older computers, it may be desirable to use a larger file size format such as Photo JPEG, or a Tiff stack sequence.

5. In order to reconstruct a z-series of three-dimensional images from a live cell recording, it is necessary to use either the up-scan or the down-scan of a z-series, but not both. 3D-DIAS incorporates a character generator to imprint the up scans and a software detection method that retains only the imprinted frames in post processing. Alternatively, this can be accomplished during acquisition by employing a shutter in the light path of the camera to omit the redundant data.
6. When a formatting issue is encountered, there are many useful tools that can mitigate these problems. QuickTime Pro can take almost any image or video format and convert it directly into a DIAS compatible QuickTime movie format (.mov). Alternatively, programs such as Graphic Converter® for Macintosh and VirtualDub® for Windows-based operating systems can provide valuable insights into the particular video or image format that is creating the issue.

The desirable number of images taken per unit time depends on the speed of movement of the specimen. For example, a fast-moving *Dictyostelium* amoebae can change its shape in a matter of seconds, so a relatively fast acquisition rate (minimum 15 frames per min (fpm)) is desirable in order to measure these changes. Recording a slow-moving cell such as a fibroblast requires fewer frames to get the same amount of data. Reducing the acquisition rate to 1 fpm is often sufficient.

7. When pouring off the supernatant of the polystyrene microspheres and Concanavalin A, it is best to use a pipet aid to gently siphon the supernatant off, as the pellet of microspheres is very easily disturbed.
8. The polystyrene microspheres and Concanavalin A solution can be kept at 4°C for up to 2 months. It then becomes necessary to rewash the beads, and resuspend them in fresh BSS. This should be done only once.
9. It may be difficult to locate a cell with a microsphere attached on the desired portion of the cell. However, as the cells crawl, it will pick up beads that are on the substrate. Therefore, choosing a cell and recording it for a long period of time, rather than looking for a cell with perfect microsphere positioning, increases the probability of capturing the microsphere in the desirable position on the cell body.
10. After approximately 40 min, the cells will have collected a large number of microspheres. This may hinder movement and change the behavior.

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Chapter 31

Mathematics of Experimentally Generated Chemoattractant Gradients

Marten Postma and Peter J.M. van Haastert

Summary

Many eukaryotic cells move in the direction of a chemical gradient. Several assays have been developed to measure this chemotactic response, but no complete mathematical models of the spatial and temporal gradients are available to describe the fundamental principles of chemotaxis. Here we provide analytical solutions for the gradients formed by release of chemoattractant from a point source by passive diffusion or forced flow (micropipettes) and gradients formed by laminar diffusion in a Zigmond chamber. The results show that gradients delivered with a micropipette are formed nearly instantaneous, are very steep close to the pipette, and have a steepness that is strongly dependent on the distance from the pipette. In contrast, gradients in a Zigmond chamber are formed more slowly, are nearly independent of the distance from the source, and resemble the temporal and spatial properties of the natural cAMP wave that *Dictyostelium* cells experience during cell aggregation.

Key words: Diffusion, Equation, Chemotaxis, *Dictyostelium*, Point source, Pipette, Zigmond chamber

1. Introduction

Chemotaxis is a vital process in a wide variety of organisms, ranging from bacteria to vertebrates. Prokaryotes use chemotaxis to move toward high nutrient concentrations or away from unfavorable conditions, while in eukaryotes it is also involved in embryogenesis, wound healing and the immune response. Chemotaxis is achieved by coupling gradient sensing to basic cell movement. Prokaryotes are too small to sense spatial gradients and therefore rely on temporal changes of the chemoattractant concentration to achieve chemotaxis. They do this by adjusting their tumbling frequency in response to temporal changes of the chemoattractant concentration ([1](#)). Eukaryotic cells are typically

large enough to be able to measure a spatial gradient. The difference in receptor occupation between each side of the cell leads to an internal polarization. A pseudopod is extended at the side with the highest receptor occupation and at the same time, pseudopod formation at all other sides is repressed, resulting in directional cell migration (2, 3).

Dictyostelium is a eukaryotic organism that is widely used to study chemotaxis (4–6). Starved *Dictyostelium* cells periodically secrete cAMP. Through relay of the cAMP signal by neighboring cells, concentric cAMP waves are generated. Starved *Dictyostelium* cells are chemotactically sensitive to cAMP and by movement in the direction of the origin of the cAMP waves the cells are able to aggregate into groups of up to 1,000,000 cells. The chemotactic response of *Dictyostelium* is optimized for the dynamic cAMP waves that coordinate both aggregation and multicellular development. Cells show a much stronger chemotactic response to a cAMP wave where the mean concentration increases over time, than to a static spatial gradient. *Dictyostelium* uses both spatial gradient sensing and the “bacterial-like” temporal gradient sensing to respond to these dynamic chemoattractant gradients (7–9).

Several chemotaxis assays have been developed that can be divided into two groups, depending on how the gradient developed. In Zigmond chambers, cells are placed on a bridge separated by a chemoattractant source and a sink reservoir (10). A gradient will be formed under the bridge, which will be nearly linear when at equilibrium. Depending on the geometry of the bridge (which in most setups is a few mm) half-maximal equilibrium is reached only after several minutes. Duncie chambers have similar properties: a linear gradient that is formed during several minutes of incubation.

Many experiments are performed with micropipettes, because this allows the precise positional stimulation of the cell (11). Since the pipette is usually in the field of microscopic observation, distances are relatively small (less than 200 μm) and equilibrium is established very fast on the order of seconds. Since a micropipette behaves like a point source, the gradient will be nonlinear approaching the equation $dC/dx = \nabla C = 1/x^2$ where x is the distance from the pipette. Thus, close to the pipette, the gradient is very steep (~100% per cell of 10 μm length at a distance of 10 μm from the pipette) while more shallow at the edge of the field of observation (10% per cell at a distance of 100 μm). In this manuscript we derive mathematical equations for the temporal and spatial properties of the gradients formed in a Zigmond chamber and delivered from a pipette. We compare the theoretical properties of these gradients with experimental data measuring the gradients using fluorescent dyes. Finally we compare

these experimental gradients with those observed during the natural aggregation of *Dictyostelium* cells.

2. Zigmond Chamber-Generated Gradients

2.1. Experimental Setup for Zigmond Chamber

Figure 1a shows the experimental setup with our modified Zigmond chamber (10). On a microscope slide, a glass bridge of ~2 mm wide and 24 mm long was placed on top of two supporting glass strips with thickness 0.15 mm. Cells were placed under the bridge to yield a density of 3×10^4 cells/cm². A block of agar containing only buffer was placed at one side of the bridge, while a block of agar containing cAMP and buffer was placed at the other side, which induces the formation of a cAMP gradient under the bridge. Cells were observed by phase contrast microscopy in an area of 350×270 μm or by confocal fluorescence microscopy in an area of 150×150 μm, both at a distance of 600–700 μm from the agar block containing cAMP.

2.2. Measurement/Analysis for Zigmond Chamber

The formation of the cAMP gradient was deduced by measuring the diffusion across the bridge of the modified Zigmond chamber using bromophenol blue ($M_w = 670$ Da). This reveals that a gradient developed during the first few minutes after the cAMP containing agar block was placed against the bridge (**Fig. 1b**). A stable linear spatial gradient was reached at 5–10 min. This spatial gradient remained approximately constant for 30 min, and then slowly diminished due to depletion of the cAMP source and accumulation of cAMP in the buffer sink (**Fig. 1c**). Thus the gradients in the modified Zigmond chamber have temporal and spatial components during the first 5 min, but stable spatial gradients during the subsequent 30 min of the experiment.

2.3. Diffusion Equations for Zigmond Chamber

When a large reservoir with cAMP is connected to another large empty reservoir through a thin bridge, diffusion will occur that can be modeled essentially with a one-dimensional diffusion equation in Cartesian coordinates:

$$\frac{\partial C(x,t)}{\partial t} = D \frac{\partial^2}{\partial x^2} C(x,t) \quad (1)$$

In this equation D (μm²/s) denotes the diffusion coefficient of cAMP, $C(x,t)$ denotes the concentration at time t (s) and position x (μm) from the source. This equation is solved with the following boundary conditions: the concentration at $x = 0$ (source) is constant with value C_s , the concentration at $x = L$ (sink) is

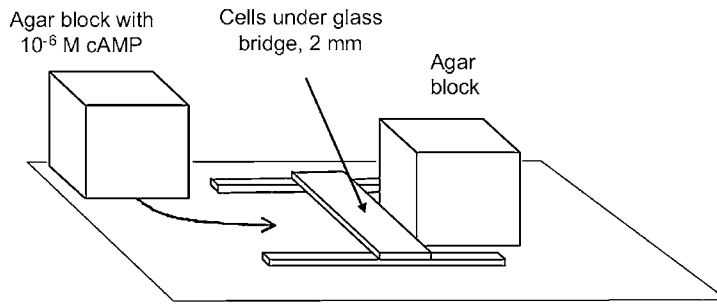
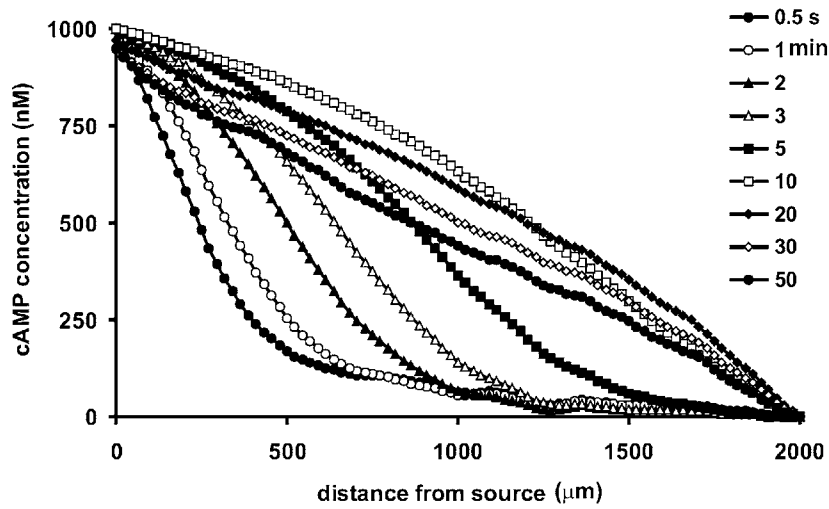
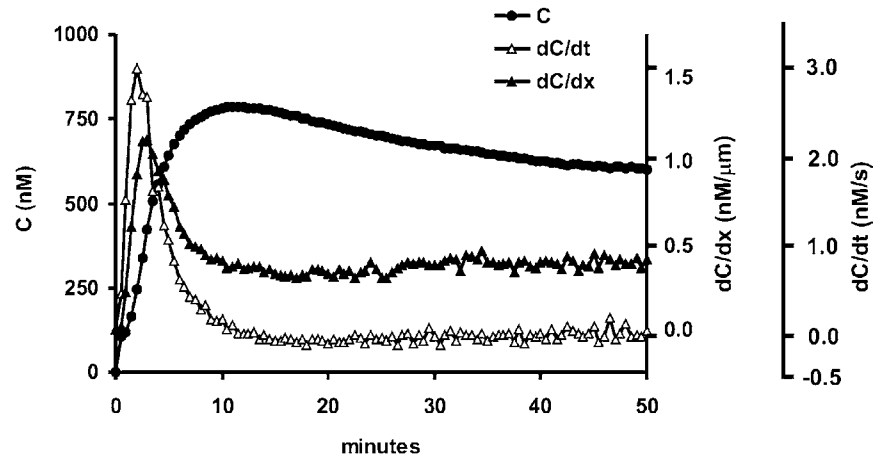
a**Chemotaxis chamber****b****c**

Fig. 1. Observed gradients in the modified Zigmond chamber. (a) Setup of the Zigmond chamber. A glass bridge of ~ 2 mm wide and 24 mm long was placed on top of two supporting glass strips with thickness 0.15 mm. Cells were placed under the bridge. A block of agar containing only buffer was placed at one side of the bridge, while a block of agar containing $1 \mu\text{M}$ cAMP and buffer was placed at the other side. (b) A gradient of cAMP develops under the bridge, visualized by diffusion of a dye added to the agar block containing cAMP. (c), gradients at the position of chemotactic observation (650–750 μm from the source). Using the local concentration of the dye we calculated the cAMP concentration C , the spatial gradient dC/dx , and temporal cAMP gradient dC/dt .

assumed to be constant and zero. L is the length of the bridge. The complete space-time solution of the concentration profile is then given by:

$$C(x, t) = C_s \left(1 - \frac{x}{L} \right) - C_s \sum_{n=1}^{\infty} 2a_n^{-1} \sin \left(a_n \frac{x}{L} \right) e^{-\frac{t}{\tau_n}} \quad (2)$$

where $a_n = \pi n$, the time constants $\tau_n = a_n^{-2} \tau_D$ and $\tau_D = L^2 / D$. The slowest time constant is τ_D / π^2 .

After some time equilibrium will appear with a time constant $\tau_D = L^2 / D$. The equilibrium concentration profile is given by the first term of **Eq. 2**:

$$C(x) = C_s \left(1 - \frac{x}{L} \right) \quad (3)$$

The gradient and the relative gradient are then given by:

$$\nabla C(x) = -\frac{C_s}{L} \quad (4)$$

and

$$\frac{\nabla C(x)}{C(x)} = -\frac{1}{L - x} \quad (5)$$

Figure 2 reveals that cAMP will diffuse from the source block approaching a steady state after about 10–20 min. The spatial gradient is initially very steep close to the source block and very shallow at the sink block (**Fig. 2b**). Over time the gradient decreases in the half of the chamber closest to the source, but increases at the other half closest to the sink. Finally a steady state is reached at 10–20 min, with a magnitude that depends on the cAMP concentration in the source and the width of the bridge. With $C_s = 1 \mu\text{M}$ cAMP as source and a bridge of $L = 2,000 \mu\text{m}$, the steady-state spatial gradient is constant at $\nabla C = 5 \text{ nM}/\mu\text{m}$. In the field of observation (600–700 μm from source) the absolute concentration C changes from 700 to 650 nM, and the relative spatial gradient from 0.77 to 0.83% across the cell. The temporal gradient profile is presented in **Fig. 2c**, showing that it reaches a maximum with magnitude and at a time that depends on the distance from the source. Close to the source the maximal temporal gradient is very high and early, and becomes lower and later further away from the source.

The experimental data of bromophenol blue diffusion (**Fig. 1b**) are in close agreement with the model, except for very long incubation times. Since the source and the sink are not indefinitely large, the concentrations in the source and sink slowly decrease and increase, respectively. As a consequence, the gradient will slowly collapse (**Fig. 1c**). At the position where the cells are observed

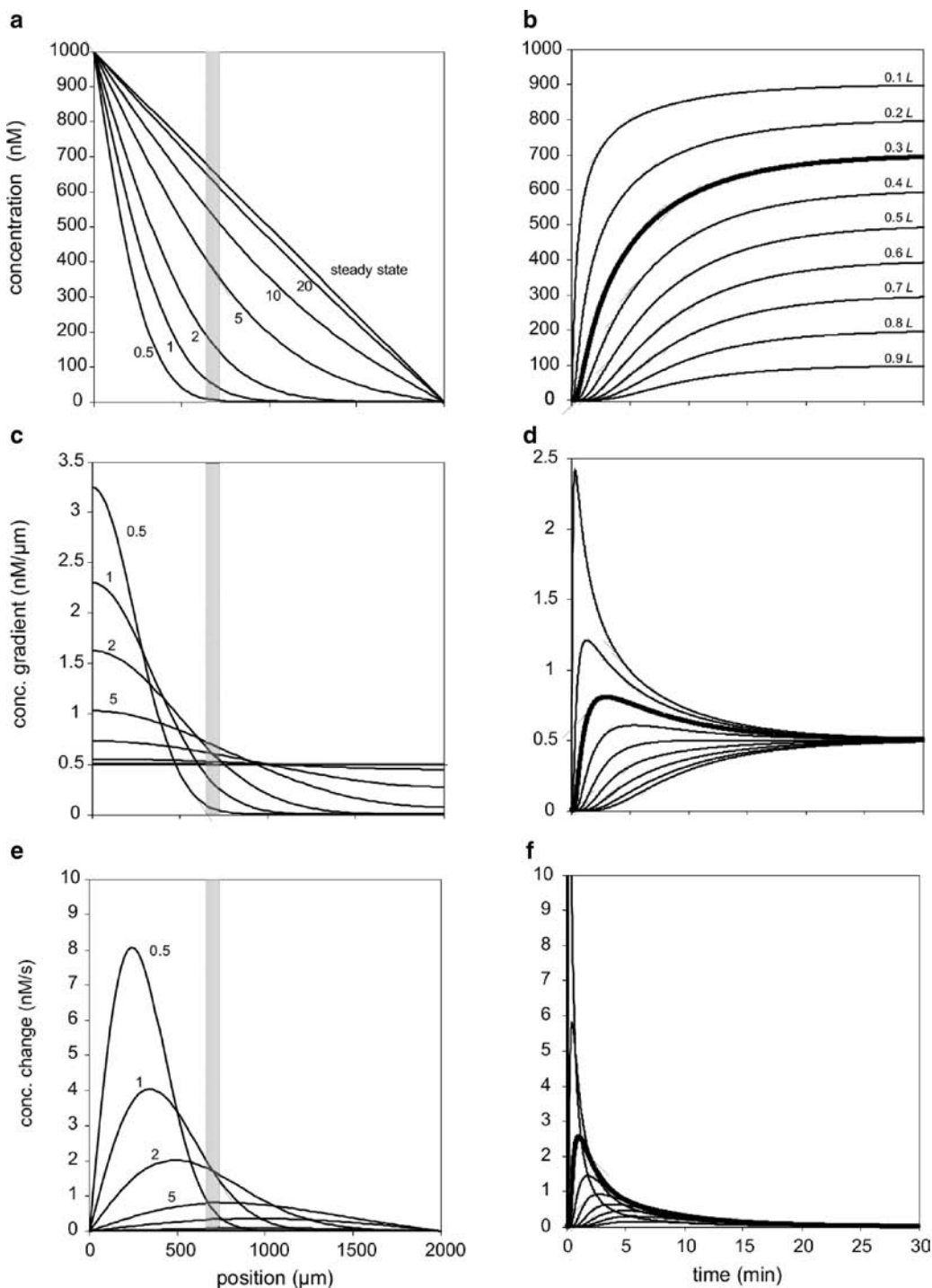


Fig. 2. Theoretical data for Zigmond chamber. **Equation 2** was used to calculate the concentration dynamics. Panels (a, d) present the concentration C . The spatial gradient dC/dx is presented in panels (b, e) and the temporal gradient dC/dt in panels (c, f), all at different positions and times from the source containing $1 \mu\text{M}$ of cAMP. The gray bars in the left panels and the thick line in the right panels indicate the place of chemotactic observation (650–750 μm from the source).

using the microscope (600–700 μm from the source), this becomes significant only after more than 2 h, much longer than the 30 min to perform the experiments. Closer to the source or the sink the gradient deviates from a linear gradient sooner (Fig. 1b). At short incubation times, the gradient is formed from 10 to 90% of the equilibrium value in about 4 min, which is only slightly slower than the formation of the cAMP wave during *Dictyostelium* cell aggregation (see later). The model and experimental data imply that the modified Zigmond chamber allows two assay conditions: during the first 5 min, the gradient is formed and therefore cells are exposed to both temporal and spatial gradients. The magnitude of these gradients resembles the temporal and spatial gradient of the natural cAMP wave during *Dictyostelium* chemotaxis (see Fig. 5 and Subheading 4). After 10 min a stable gradient is formed without a temporal component.

3. Micropipette-Generated Gradients

3.1. Experimental Setup for Pipette Assay

For the micropipette assay, a droplet of a cell suspension was placed on a glass slide yielding a cell density of 5×10^4 cells/cm²; the droplet has a diameter of about 10 mm and a height of 3 mm. After the cells were allowed to adhere, a pipette filled with 100 μM cAMP is placed just above the glass surface. cAMP was delivered from the femtotip at a pressure ranging from 0 to 100 hPa.

3.2. Measurement/Analysis for Pipette Assay with and Without Flow

The formation of the cAMP gradient was deduced by measuring the diffusion of the fluorescent dye lucifer yellow ($M_w = 457$ Da) using confocal microscopy. The fluorescence intensity at different distances from the pipette was recorded in pixel elements (0.404×0.404 μm) using excitation at 488 nm and a 520–550 nm band pass filter for the emission. The data were calibrated using the fluorescence intensity of diluted lucifer yellow added homogeneously to the bath (Fig. 3a). The deduced cAMP concentration profile (Fig. 3b) reveals a steep gradient in the vicinity of the pipette that rapidly declines at longer distances from the pipette. This gradient was stable within 20 s after application of the pipette. The concentration at the tip of the pipette is only 150 nM, compared to the 100 μM cAMP inside the pipette.

The amount of cAMP released from the pipette can be increased by applying pressure to the pipette, which induces a steady flow of cAMP that diffuses away. The cAMP concentration profile was again deduced from the pipette containing lucifer yellow, revealing that the concentration at the tip increases from 150 nM cAMP without pressure to 3,000 nM cAMP at a

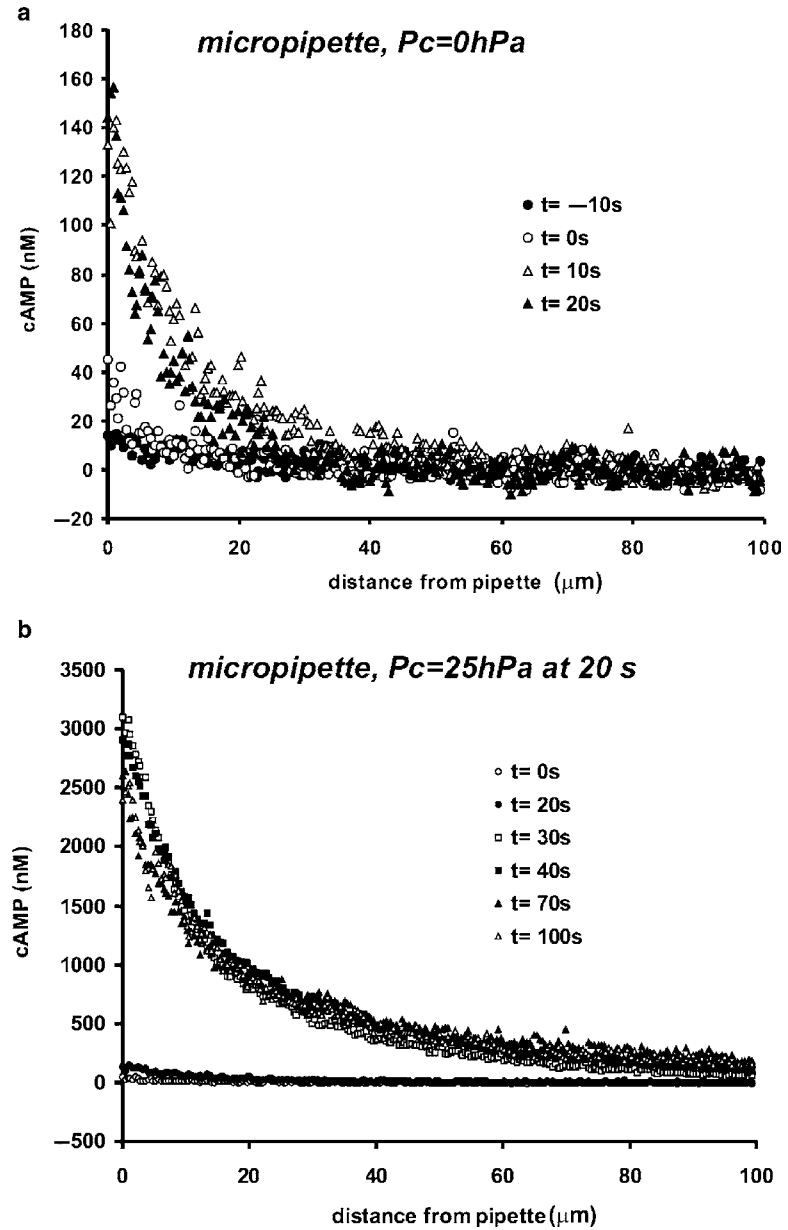


Fig. 3. Observed gradients in the micropipette assay. A micropipette filled with $100\text{ }\mu\text{M}$ cAMP and lucifer yellow was applied just above the glass surface in a droplet of cells. The fluorescence intensity was measured by confocal microscopy at different times after positioning of the pipette in the fluid. Initially no pressure was applied to the pipette (a), and a pressure of 25 hPa was applied at 20 s (b). The concentration of cAMP was deduced using a dilution series of lucifer yellow added homogeneously to the bath.

pressure of 25 hPa (Fig. 3c). Similar to the case without applied pressure, the concentration rapidly decreases with distance from the pipette, except close to the pipette (within $2\text{ }\mu\text{m}$) where the concentration remains relatively uniform.

3.3. Diffusion Equations for Pipette Gradients Without Flow

When a pipette filled with cAMP is positioned just above the floor of a chamber, cAMP will diffuse effectively in a half-sphere. The point of the pipette is in the center of the sphere; the opening of the pipette with opening r_0 is regarded as a small sphere from which cAMP diffuses. The diffusion equation is then given in sphere coordinates by:

$$\frac{\partial C(x,t)}{\partial t} = D \frac{1}{x^2} \frac{\partial}{\partial x} x^2 \frac{\partial}{\partial x} C(x,t) \quad (6)$$

In this equation D ($\mu\text{m}^2/\text{s}$) denotes the diffusion coefficient of cAMP, $C(x,t)$ denotes the concentration at time t (s), and distance x (μm) from the pipette. We assume that the gradient in the pipette builds up very fast. Hence, equilibrium in the small sphere at the tip of the pipette will be reached rapidly leading to a constant concentration C_s at the tip. Numerical analysis of diffusion in the pipette and the small sphere suggests that the time constant of this process is less than 1 s. In addition, measurements presented in **Fig. 3b** reveal that equilibrium at the tip is reached within 10 s. We use the boundary condition that the concentration at the edge of the bath, $x = R$ is constant and zero. The complete space-time solution is then given by:

$$C(x,t) = C_s \frac{r_0}{x} \frac{R-x}{R-r_0} - C_s \frac{r_0}{x} \sum_{n=1}^{\infty} 2a_n^{-1} \sin\left(a_n \frac{x-r_0}{R-r_0}\right) e^{-\frac{t}{\tau_n}} \quad (7)$$

where $a_n = \pi n$, the time constants $\tau_n = a_n^{-2} \tau_D$ and $\tau_D = (R-r_0)^2 / D$.

A very good approximation for **Eq. 7** is:

$$C(x,t) = C_s \frac{r_0}{x} \text{erfc}\left(\frac{1}{2} \frac{x-r_0}{\sqrt{Dt}}\right) \quad (8)$$

The time needed to reach this equilibrium strongly depends on the distance from the pipette $\tau_D = (x-r_0)^2 / D$; when t equals this time constant about half-maximal equilibrium is reached.

After some time the gradient reaches equilibrium. The equilibrium concentration profile is given by the first term of **Eq. 7**, which for a large bath ($R \rightarrow \infty$) and $x > r_0$ is given by:

$$C(x) = C_s \frac{r_0}{x} = \frac{\alpha C_p}{x} \quad (9)$$

where C_p is the cAMP concentration in the pipette. The absolute spatial gradient and the relative spatial gradient are then given by:

$$\nabla C(x) = -C_s \frac{r_0}{x^2} = -\frac{\alpha C_p}{x^2} \quad (10)$$

and

$$\frac{\nabla C(x)}{C(x)} = -\frac{1}{x} \quad (11)$$

The experimental equilibrium data (**Fig. 4a**) were fitted using **Eq. 9** showing that they are in close agreement with the calculated

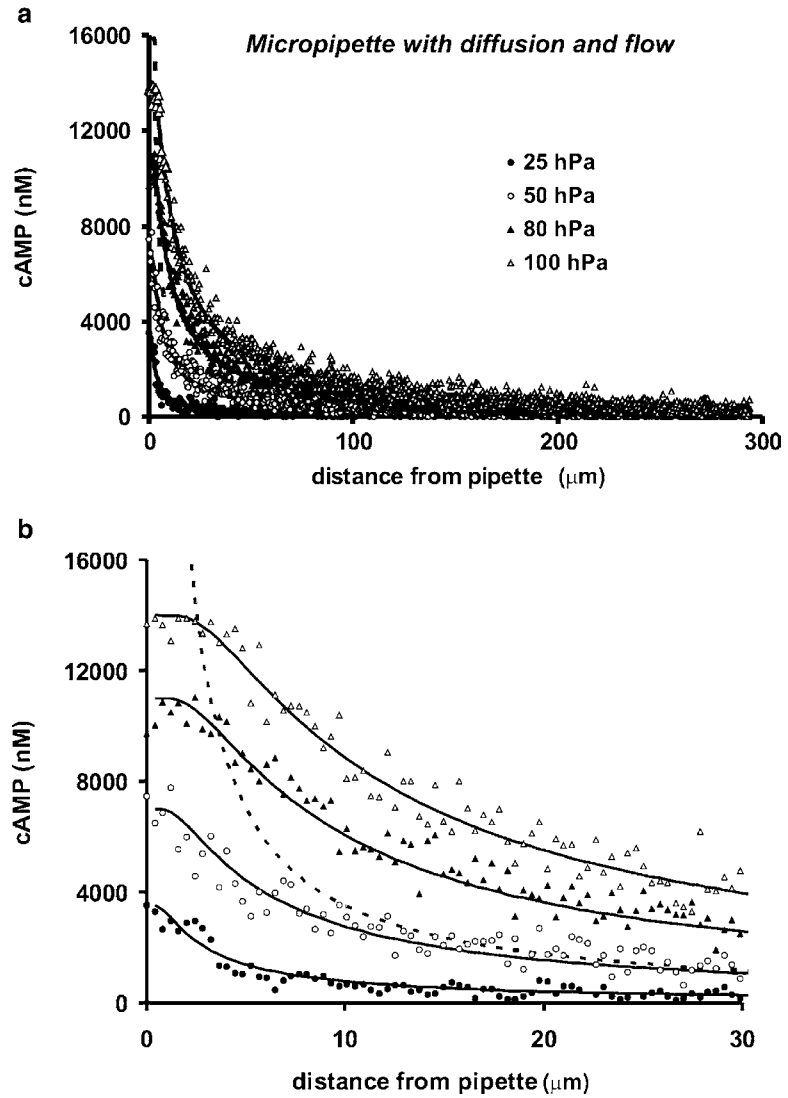


Fig. 4. Experimental equilibrium data of the cAMP gradient with different flow from the micropipettes. A micropipette filled with cAMP and lucifer yellow was applied just above the glass surface in a droplet of cells. The pressure applied was 25, 50, 80, and 100 hPa. The equilibrium fluorescence intensity was measured by confocal microscopy at 30 s after application of the pipette. The *lines* are the fitted data using **Eq. 13** with C_s and F as indicated in **Table 1**; the *dashed line* is **Eq. 17** for 50 hPa. The *lower panel (b)* shows the same data as *upper panel (a)*, but only at shorter distance from the pipette.

gradient profile, except very close to the pipette. The experiment reveals that the measured cAMP concentration at the tip (i.e., C_s) is only 150 nM compared to 100 μM in the pipette, indicating that a very strong gradient is formed in the pipette.

3.4. Diffusion Equations for Pipette Gradients with Flow

When pressure is applied to the pipette, liquid will flow out of the pipette with flux F ($\mu\text{m}^3/\text{s}$). To account for the flow the diffusion equation has to be extended with convection:

$$\frac{\partial C(x, t)}{\partial t} = D \frac{1}{x^2} \frac{\partial}{\partial x} x^2 \frac{\partial}{\partial x} C(x, t) - \frac{F}{2\pi x^2} \frac{\partial}{\partial x} C(x, t) \quad (12)$$

A complete space-time solution of this equation is difficult to obtain. However, the equilibrium solution can be obtained relatively easy. For a large bath the steady-state concentration profile is given by:

$$C(x) = C_s \frac{1 - e^{-\frac{F}{2\pi Dx}}}{1 - e^{-\frac{F}{2\pi D r_0}}} \quad (13)$$

The absolute spatial gradient and the relative gradient are then given by:

$$\nabla C(x) = -C_s \frac{F}{2\pi Dx^2} \frac{e^{-\frac{F}{2\pi Dx}}}{1 - e^{-\frac{F}{2\pi D r_0}}} \quad (14)$$

and

$$\frac{\nabla C(x)}{C(x)} = -\frac{F}{2\pi Dx^2} \frac{e^{-\frac{F}{2\pi Dx}}}{1 - e^{-\frac{F}{2\pi D r_0}}} \quad (15)$$

The results shown in **Figs. 3** and **4** reveal that with an increase of pressure from 0 to 25 hPa, the concentration at the tip of the pipette increases substantially from 150 nM at 0 hPa to 3,000 nM at 25 hPa. In the absence of pressure, the concentration at the tip is very low due to limited diffusion in the narrow tip of the pipette, causing a large concentration gradient inside the pipette. With liquid flow, the liquid at the tip is replaced by interior liquid of higher concentration, resulting in a higher cAMP concentration at the tip.

The gradient profiles at different applied pressures (**Fig. 4**) were fitted using **Eq. 13**, yielding the values for the concentration at the tip (C_s) and flow (F) as presented in **Table 1**. The calculated lines are in very good agreement with experimental data, both close to the pipette and at longer distances from the pipette. This suggests that we derived an accurate model for gradient formation from a pipette with diffusion and flow.

Table 1
Values of F and C_s obtained by fitting experimental observations in Fig. 4 with Eq. 13

Applied pressure (P_c , hPa)	Fitted flow (F , $\mu\text{m}^3/\text{s}$)	Fitted concentration at tip (C_s , nM)	$\alpha = \frac{C_s}{C_0} \frac{F}{2\pi D}$ (μ/m)
25	15.000	3,500	0.0875
50	30.000	7,000	0.35
80	48.000	11,000	0.88
100	60.000	14,000	1.4

Using the observed data and fitted values of F and C_s , Eq. 13–15 can be simplified. The calculated flow is large relative to $2\pi D r_0$, which implies that the denominator in Eq. 13–15 reduces to 1. Furthermore, at longer distances from the pipette (i.e., at large x), $F/2\pi D x$ in Eq. 13 becomes very small, and consequently Eq. 13 reduces to the following equation:

$$C(x) = C_s \frac{F}{2\pi D x} \quad (\text{for large } x) \quad (16)$$

This equation has the same form as Eq. 9:

$$C(x) = \frac{\alpha C_p}{x} \quad (17)$$

where $\alpha = \frac{C_s}{C_p} \frac{F}{2\pi D}$ in $(\mu\text{m})^{-1}$

In Fig. 4 the dashed line represents Eq. 17 with the experimentally fitted values of F and C_s , showing that the experimental data are very well described with the simple Eq. 17 at distances beyond about 15 μm from the pipette. Finally, inspecting Table 1, we noted that the fitted values of both C_s and F increase linearly with the applied pressure P_c , indicating that α , and thus the absolute concentration, depends on P_c^2 .

The usual field of observation is $100 \times 100 \mu\text{m}$ with the pipette placed somewhere in the field. The time constant to reach equilibrium, $\tau_D = (x - r_0)^2 / D$, indicates that in the field of observation (x maximal 100 μm ; $r_0 = 0.5 \mu\text{m}$, $D = 1,000 \mu\text{m}^2/\text{s}$) equilibrium is reached within 10 s after application of the pipette, as was observed experimentally (Fig. 3). Thus gradients from pipettes are essentially stable spatial gradients, except at very long distances from the pipette.

4. Gradients Generated by Aggregating *Dictyostelium* Cells

Dictyostelium cells secrete cAMP in a pulsatile manner with a periodicity of about 5 min. The cAMP signal is relayed through the field leading to waves of cAMP that propagate with a velocity of about 300 $\mu\text{m}/\text{min}$. These waves have been visualized by fluorography using competition between secreted cAMP and added [^3H]cAMP for binding to a cAMP-binding protein.

We calculated the extracellular cAMP concentration during natural cell aggregation using the original fluorographs made by Tomchik and Devreotes (12) as presented in (13). The fluorographs represent the competition of a fixed amount of [^3H]cAMP and secreted cAMP for binding to the regulatory subunit of cAMP-dependent protein kinase. In essence, this experiment is an isotope dilution assay in space that follows the equation:

$$A(x) = a \left[\frac{C_0 - bl}{C_x - bl} - 1 \right] \quad (18)$$

where $A(x)$ is the cAMP concentration at position x , C_0 is the amount of [^3H]cAMP-binding in the absence of cAMP, bl is the blank of the assay (i.e., the amount of [^3H]cAMP-binding in the presence of excess cAMP), C_x is the amount [^3H]cAMP-binding at position x competed by unknown amount of cAMP, and a is a proportionality constant that is determined by measuring the amount [^3H]cAMP-binding at known amounts of unlabelled cAMP. For cAMP determinations in a test tube, the measured units are counts per minute (cpm), while in fluorographs the units are in gray scales. We determined the proportionality constant a in a test tube (550 nM), and estimated the values of C_0 and bl from the information provided by Devreotes et al. (13). C_0 is the gray level in the absence of cAMP (127 in Fig. 2 from ref. 13), while bl is the gray level in the presence of saturating cAMP (90 in Fig. 2 from ref. 13). The cAMP concentration in natural waves was calculated as the average of two successive waves. From the spatial resolution and the speed of wave propagation we calculated the spatial cAMP gradient and the temporal cAMP gradient during cell aggregation.

The results (Fig. 5) show that the extracellular cAMP concentration profiles are approximately symmetric cAMP waves. The width at the base of the wave is about 1,600 μm . Since the wave travels at a speed of about 300 $\mu\text{m}/\text{min}$, the periodicity of the wave is approximately 5–6 min. The rising flank of the wave, as well as the width at half-maximal concentration, is about 450 μm or 1.5 min. The absolute spatial gradient of the wave, $\nabla C = dC/dx$, increases during the rising flank of the wave and reaches a maximum of about 4 nM/ μm at about 1 min after arrival

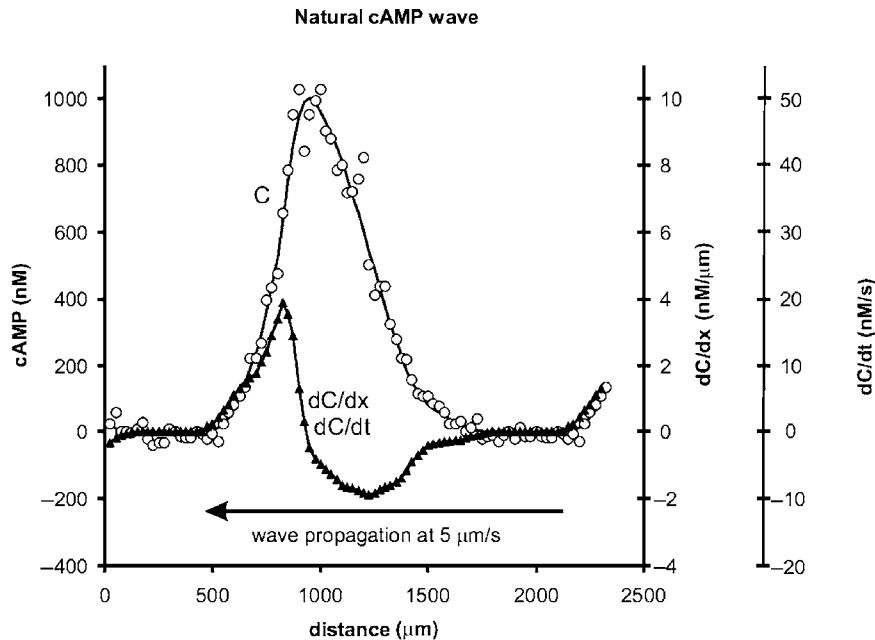


Fig. 5. Natural cAMP wave during cAMP aggregation. The cAMP concentration was deduced from fluorographs of released cAMP measured by (13). For calculations see part 4. Gradients Generated by Aggregating *Dictyostelium* Cells the wave of CAMP travels through a field of cells at a rate of about 300 $\mu\text{m}/\text{min}$ (5 $\mu\text{m}/\text{s}$).

of the cAMP wave. This absolute spatial gradient is only twofold larger than the maximal spatial gradient in the Zigmond chamber of 1.8 $\text{nM}/\mu\text{m}$ (see Fig. 5). The relative steepness of the wave, $\nabla C/C$ is around $0.007 (\mu\text{m})^{-1}$ during most of the rising flank of the cAMP wave (i.e., 7% concentration difference between front and back of a 10-mm long cell).

The cAMP waves are propagated in the field of cells with a speed $v = dx/dt$ of 300 $\mu\text{m}/\text{min}$ (13). Therefore, the temporal gradient is given by $dC/dt = v \nabla C$. Thus, the temporal gradient follows the spatial gradient and reaches a maximum value of about 17.5 nM/s at 1-min after arrival of the cAMP wave. In the Zigmond chamber the temporal gradient reaches a maximum of 3 nM/s at 2 min after application of cAMP (Fig. 1c).

5. Conclusions

The gradients formed in the modified Zigmond assay are very different from the gradients formed by a point source releasing a constant flux of cAMP. The major differences are: a temporal-spatial

gradient with little distance dependency in the Zigmond assay versus a stable gradient with very strong distance dependency. It is surprising that the distance dependency of the pipette assay is often not taken into account, exemplified by the absence of information on the distance from the pipette where the chemotaxis data were obtained, a situation we also were not aware of in the past (14). Chemotaxis in *Dictyostelium* is mainly dependent on the absolute spatial gradient resulting in only about 10 occupied receptors more at the front of the cells relative to the rear of the cell at threshold conditions, at prevailing receptor occupancies of around 1,000 receptors. It will be a major effort to uncover how cells are able to deduce spatial information from a signal that is inherently very noisy due to the high average receptor occupancy. The gradient models presented here may help to design experiments to deduce the fundamental principles of gradient sensing and directed locomotion.

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Chapter 32

Modeling Spatial and Temporal Dynamics of Chemotactic Networks

Liu Yang and Pablo A. Iglesias

Summary

When stimulated by chemoattractants, eukaryotic cells respond through a combination of temporal and spatial dynamics. These responses come about because of the interaction of a large number of signaling components. The complexity of these systems makes it hard to understand without a means of systematically generating and testing hypotheses. Computer simulations have proved to be useful in testing conceptual models. Here we outline the steps required to develop these models.

Key words: Mathematical model, Chemotaxis, Reaction–diffusion, Systems biology, Virtual cell

1. Introduction

What does a mathematical model tell me that I didn't know before? This is a common question posed by experimenters, suspicious that models merely package already-known information and provide few new insights. Models can do two things. First, they can “verify that known interactions in some system can produce the observed qualitative behavior” (1). When employed this way, models act mostly as tools providing a form of consistency check, ensuring that the posited conceptual models behave as they are supposed to. However, the real benefit of models is a predictive tool. In this case, models usually precede complete knowledge of the system but serve to channel experimental investigations.

It is usually thought that the introduction of mathematical and computational techniques in the study of signaling pathways is a relatively new phenomenon – an offshoot of the

advent of Systems Biology. And, while it is true that recent years have seen a veritable blossoming in the use of computational models in cell biology, it is also worth remembering that mathematical models have a long history of making significant contributions to the understanding of biological systems. The best such example is the use of models by Hodgkin and Huxley to understand electrophysiology.

Mathematical models of bacterial chemotaxis (reviewed in (2)) have a strong record of successfully leading to predictions that were later confirmed experimentally. Macnab and Koshland (3) were the first to use theoretical means to postulate a signaling mechanism that accounted for the adaptive behavior observed in the run/tumble behavior of bacteria (Fig. 1). Their “temporal gradient apparatus” consists of two enzymes catalyzing the synthesis (enzyme 1) or degradation (enzyme 2) of a compound. Both enzymes are activated after chemoattractant receptor activation, albeit at different rates: fast for enzyme 1, slow for enzyme 2. In a time-varying gradient of increasing chemoattractant concentration, the net result is an increase in the concentration of the compound. Though presented as a conceptual model, it later led Koshland to posit that the enzymes could be regulated through methylation – a prediction that later proved to be true (4). Another example

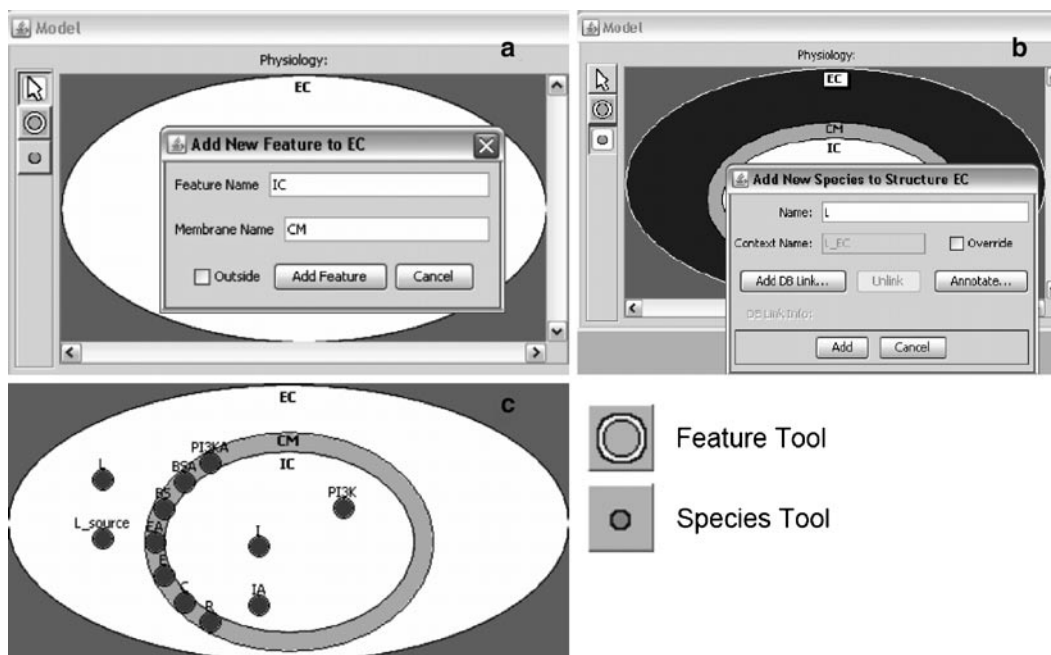


Fig. 1. *Specifying the components.* (a) Adding a new feature inside the EC (Extracellular) compartment, with feature name IC (Intracellular), and membrane name CM (cell membrane). (b) Adding a new molecular species to the structure EC, with name L (ligand). (c) All the molecular species are added to associated compartments.

of theory leading experimentation is the proposal by Barkai and Leibler (5) that the bacterial signaling mechanism is robust (verified later (6)) or that of Bray et al. (7) that the sensing capabilities could be increased if receptors worked cooperatively, in such a way that ligand-mediated changes in the activity of a receptor would propagate to neighboring receptors in a cluster – a model for which there is now experimental evidence (8).

It is perhaps not surprising, owing to the successful marriage between models and experiments in bacterial chemotaxis, that mathematical modeling has quickly become an accepted tool for studying eukaryotic chemotactic networks (reviewed in (9)). In this chapter we outline the steps necessary for developing these models. We begin by presenting some necessary theoretical background.

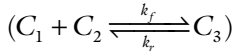
1.1. Modeling Temporal Dynamics

When developing a model describing the temporal dynamics of a biochemical network we use ordinary differential equations (ODEs). For a network with n interacting species, labeled $C_1, \dots, C_n$, we describe temporal changes in the concentration of species i , denoted by C_i , through the differential equation:

$$\frac{dC_i(t)}{dt} = f(C_1, \dots, C_n). \quad (1)$$

The function f specifies the interconnections that affect the concentration of C_i . The specific form of this function depends on the assumptions made about the biochemical interaction. We consider two types of reactions: Binding reactions and enzymatic reactions.

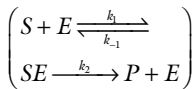
1.1.1. Binding Reaction



Each time the forward reaction occurs, molecules of species C_1 and C_2 bind together to create one molecule of C_3 . With the reaction affinity of k_f , we can use a flux term $J_f = k_f \times C_1 \times C_2$ to represent the rate of consumption of C_1 and C_2 , which is also the rate of production of C_3 . The reverse reaction occurs with affinity k_r , with one unit of C_3 decomposing into one unit of C_1 and one unit of C_2 . The flux $J_r = k_r \times C_3$ represents the rate of the reverse reaction. The whole reaction therefore occurs at the combined rate of:

$$\frac{dC_3}{dt} = -\frac{dC_1}{dt} = -\frac{dC_2}{dt} = J_f - J_r = k_f C_1 \times C_2 - k_r C_3.$$

1.1.2. Enzymatic Reaction Using Michaelis– Menten Dynamics



In enzymatic reactions, the enzyme E catalyzes the conversion of the substrate S into product P . The substrate S and enzyme E reversibly form an intermediate SE that is converted to product P with affinity $k_2 \times E$. The standard assumption is that the enzyme turnover is so fast that the concentration of enzyme–substrate intermediate does not change during the reaction: $dSE/dt = 0$. This reaction occurs at the rate of:

$$\frac{dP}{dt} = \frac{V_{\max} \times S}{K_m + S}.$$

where $V_{\max} = k_2 E_{\text{Tot}}$ and $K_m = (k_{-1} + k_2)/k_1$.

1.2. Modeling Spatial Dynamics

The most significant difference between the observed chemoattractant-induced behavior of bacterial and eukaryotic cells is that the latter can interpret gradients spatially. This is observed experimentally in that gradients elicit spatially segregated distributions of intracellular markers when exposed to chemoattractant gradients (10). Modeling the concentration of a biochemical species C_i that depends on time, t , and spatial dimension, x , requires the use of partial differential equations (PDE). In particular, biochemical models using reaction–diffusion equations are needed:

$$\frac{\partial C_i(x, t)}{\partial t} = f(C_1, \dots, C_n) + D \frac{\partial^2 C_i(x, t)}{\partial x^2} \quad (2)$$

As before, the function f specifies the interconnections that affect the concentration of C_i . The right-hand most term in the PDE specifies the diffusion of the species. The variable D is the diffusion coefficient. Its value can range around $10 \mu\text{m}^2/\text{s}$ for proteins in the cytosol, $1 \mu\text{m}^2/\text{s}$ for lipids in the membrane, and $0.1 \mu\text{m}^2/\text{s}$ for proteins in the membrane (11).

To solve for $C_i(x, t)$ requires that an initial distribution, $C_i(x, 0)$, be known. It is also necessary to have boundary conditions. For PDEs, there are several available choices. The most common form of boundary condition is to specify the flux of the species at the boundary.

In modeling spatial dynamics, it is sometimes necessary to consider situations where the interacting species reside on different compartments. For example: binding can occur when one reactant resides on the cell membrane (a binding site) and the other in an extra- or intracellular volume (e.g., the cytosol). In this case, the product is on the membrane.

1.3. LEGI Model of Gradient Sensing

As an example of how to create a computational model of a chemosensory system we use a simplified model of gradient sensing, referred to as the LEGI (local excitation, global inhibition) model (9). This model can be viewed as an extension of the Macnab and Koshland model (3) in that receptor occupancy triggers two types of signals: a fast excitation and a slow inhibition that together regulate an observable response. Where the model differs is that it assumes different spatial localizations: the excitation is local; the inhibition, global. When the spatial location is not an issue, for example, when the cell is stimulated by a homogeneous chemoattractant stimulus, the response is, as in the bacterial case,

adaptive. However, in the presence of a chemoattractant gradient, the LEGI model leads to a spatial response. In this chapter we assume that this response regulates binding sites for phosphoinositide 3-kinase (PI3K). This is part of a model that combines parallel LEGI mechanisms to regulate the phospholipid signaling observed in *Dictyostelium* cells (12).

2. Materials

2.1. Compartmental Models

In general, the right-hand side of Eqs. 1 and 2 is a nonlinear function of the species' concentrations. As such, analytic solutions are rarely available. It is thus necessary to solve this equation numerically, requiring a numerical simulation package. The most popular, general-purpose packages are Matlab (Mathworks, Natick, MA), Mathematica (Wolfram, Champaign, IL), and Maple (Maplesoft, Waterloo, Canada). All are relatively easy to use and provide great functionality. Alternatively, a number of simulation packages specifically tailored to the biological signaling community have appeared (reviewed in (13)). In most of these, a graphical user interface allows the user to specify a biochemical interaction by selecting the type of reaction and the kinetic coefficients. The package then automatically generates and solves the necessary ODEs.

2.2. Spatial Models

For spatially varying models, the list of available simulation packages is considerably smaller. Comsol Multiphysics (Comsol, Burlington, MA), a general-purpose simulation package originally designed to work with Matlab but now independent, allows the user to specify general PDEs, or to select from one of several predefined forms, including reaction–diffusion equations such as Eq. 2. The solution is obtained using finite-element methods (14).

The Virtual Cell is one of the few simulation packages specially tailored to cell biology that can deal with spatially varying simulations. Unlike most other software packages that reside and carry out the simulations in the user's computer, the Virtual Cell software is maintained at a central server within the National Resource for Cell Analysis and Modeling (NRCAM) at the University of Connecticut Health Center (15). Using the Virtual Cell is done through a Java application over the internet. Use of the Virtual Cell requires an account, available at <http://www.nrcam.uchc.edu/login/login.html>. Funded through the National Center for Research Resources, a component of the National Institutes of Health, the Virtual Cell is free for users in an academic environment. General purpose tutorials are available (16).

3. Methods

In this chapter, we use the LEGI model of gradient sensing as an example to demonstrate how these processes can be modeled mathematically and connected together in simulation to produce gradient sensing. We use the Virtual Cell as our modeling environment (*see* [Note 1](#)). The reactions modeled in the LEGI model are listed in [Table 1](#); the molecules involved, as well as their spatial distribution in the cell geometry, are specified in [Table 2](#).

A complete model in Virtual Cell consists of three components:

1. A Biomodel component where we specify: the molecular species involved in the biological model, the physiological compartments in which they reside, and their interactions.
2. A Geometry component that allows for specifying the shapes and dimensions of each compartment.
3. A Mathematical component based on the Virtual Cell Math Description Language (VCMDL) that allows for access of mathematical formulae behind the biological model.

To create a biological model in Virtual Cell, it is necessary to define both the Biomodel component and the Geometry component. Equations in the Mathematical component are generated automatically but are also available for manual editing.

3.1. Specifying the Components

1. When Virtual Cell is started, a new instance of Biomodel opens, allowing the creation of a new model ([Fig. 1](#)).
2. We start by double clicking on the unnamed compartment, and naming it “EC” (for extracellular).
3. To add a cellular compartment, use the Feature Tool by clicking the icon, then click the EC compartment. You now specify the name of the cell’s intracellular (IC) and cell membrane (CM) ([Fig. 1a](#)). We will only need this one cellular compartment to create the LEGI gradient sensing model.
4. Next, add all the molecular species to the model by using the Species Tool ([Fig. 1b](#)). To start, click the icon followed by the EC compartment, and name the new species “L” (for Ligand). Click Add. Similarly, click on the Membrane and Cytosol compartments to add in molecular species that belong in each. [Table 2](#) lists the necessary molecular species and their respective compartments.
5. When all the molecular species are added ([Fig. 1c](#)), we can proceed to define interactions between them. Each reaction is defined in a specific compartment. Reactions in a volume can only involve molecules from the same volume, but reactions on the membrane can involve molecules from the membrane and neighboring compartments.

Table 1
List of reactions

Reaction	Description	Reaction type	Reaction rate (#/($\mu\text{m}^2\cdot\text{s}$))	k_f (units)	k_r (units)
1 $L_{\text{source}} \leftrightarrow L$	Diffusion of cAMP from source	(see Note 6)	$k_f \times L_{\text{source}} - k_r \times L$	1 (#/($\mu\text{m}^2\cdot\mu\text{M}\cdot\text{s}$))	1 (#/($\mu\text{m}^2\cdot\mu\text{M}\cdot\text{s}$))
2 $L + R \leftrightarrow C$	cAMP binding to CAR1	Binding	$k_f \times L \times R - k_r \times C$	1.66 ($\mu\text{M}\cdot\text{s}$) ⁻¹	0.39 (s ⁻¹)
3 $E + C \rightarrow EA + C$	Receptor-mediated local excitation	Enzymatic	$k_f \times E \times C - k_r \times EA$	0.001 ($\mu\text{m}^2/(\# \cdot \text{s})$)	0.1 (s ⁻¹)
4 $I + C \rightarrow IA + C$	Receptor-mediated global inhibition	Enzymatic	$k_f \times I \times C - k_r \times IA$	0.01 ($\mu\text{M}\cdot\text{s}$) ⁻¹	10 (#/($\mu\text{m}^2\cdot\mu\text{M}\cdot\text{s}$))
5 $BS + EA \rightarrow BSA, BSA + IA \leftrightarrow BS$	LEGI-mediated activation of binding site for PI3K	Enzymatic	$k_f \times BS \times EA - k_r \times BSA \times IA$	0.01 ($\mu\text{m}^2/(\# \cdot \text{s})$)	1,000 ($\mu\text{M}\cdot\text{s}$) ⁻¹
6 $PI3K + BSA \leftrightarrow PI3KA$	PI3K binding to binding sites	Binding	$k_f \times PI3K \times BSA - k_r \times PI3KA$	2 ($\mu\text{m}^2/(\# \cdot \text{s})$)	1 (s ⁻¹)

Table 2
List of species

Molecule	Description	Compartment	Initial condition	(units)	Diffusion ($\mu\text{m}^2/\text{s}$)
L_source	Chemoattractant concentration at the source	EC	Variable		300
L	Chemoattractant concentration	EC	Variable		300
R	Unoccupied receptor	CM	100	$\#/\mu\text{m}^2$	
C	Occupied receptor	CM	0	$\#/\mu\text{m}^2$	
E	Excitation substrate	CM	100	$\#/\mu\text{m}^2$	
EA	Local excitation signal	CM	0	$\#/\mu\text{m}^2$	
I	Inhibition substrate	IC	0.1	μM	13
IA	Global inhibition signal	IC	0.1	μM	13
BS	Inactive PI3K-binding site	CM	100	$\#/\mu\text{m}^2$	
BSA	Activated PI3K-binding site	CM	0	$\#/\mu\text{m}^2$	
PI3K	Free, cytosolic PI3K	IC	0.1	μM	10
PI3KA	Bound, activated PI3K	CM	0	$\#/\mu\text{m}^2$	

3.2. Specifying the Reactions

1. We start by defining the membrane binding reaction between the extracellular ligand (L) and the receptors (R) on the cell membrane. As this reaction includes molecules in both the membrane and extracellular compartments, it takes place in the membrane compartment. Right click on the cell membrane compartment, and choose the “Reactions...” option. This will open a new window (**Fig. 2a**) where all membrane reactions can be defined. This window is divided into three sections, representing the EC (extracellular compartment), CM (cell membrane), and IC (intracellular compartment). All available molecules are displayed in the appropriate compartments.
2. To add a new reaction, click the reaction icon, and click in the middle region representing the Membrane compartment. Click the connection icon to link the molecular players to this reaction. Draw lines from the species to the reaction. As the line is drawn and the mouse hovers on the reaction site, three possible names: “reactant,” “product,” and “catalyst” can appear. Choose the proper category to end the line.

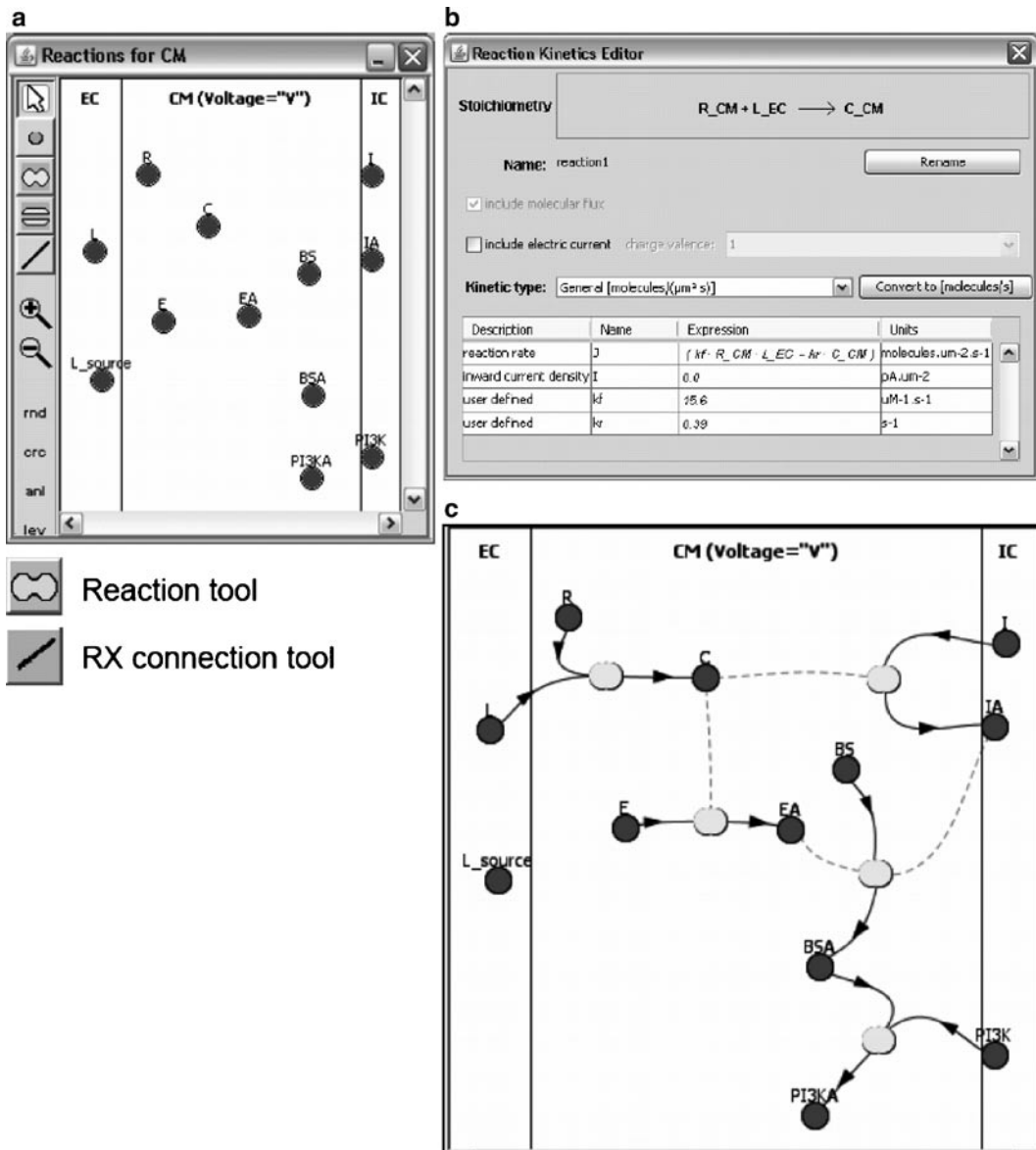


Fig. 2. *Specifying the reactions.* (a) Molecular species are divided into three adjacent regions, *EC* (extracellular), *CM* (cell membrane), and *IC* (intracellular). Reactions can be specified in any of these three compartments. (b) In the “Reaction Kinetic Editor,” we specify the reaction rate expression, as well as the kinetic reaction rates associated with the expression. (c) In this model, the reactions are defined in *CM*. Solid lines going from molecular species to reaction sites connect reactants; solid lines going from reaction sites to molecular species connect products; and dotted lines connect enzymes to reaction sites.

- Double click on the reaction to open the “Reaction Kinetics Editor” (Fig. 2b). Choose the kinetic type as “General [molecules/(\(\mu\text{m}^2\text{s}\))]” for the purpose of this example (see Note 2). Type in

the expression for the reaction rate J (*see* [Note 3](#)). For the ligand membrane-binding reaction, this expression is:

$$J = k_f \times R\_CM \times L\_EC - k_r \times C\_CM.$$

When this expression is defined, the software automatically recognizes previously undefined variables, namely the kinetic rate constants in this case. We can now specify the rate constants “ k_f ” and “ k_r ” of this reaction. As we are not modeling any membrane currents, the variable “ I ” can be ignored.

4. To define a catalytic reaction, simply adjust the reaction rate expression “ J ” to include the catalytic activities of the enzymes involved. We proceed to define all reactions listed in [Table 2](#). [Figure 2c](#) shows a graphical representation of all reactions in the CM compartment.
5. Save this model as “LEGI.”

3.3. Specifying the Geometry

1. To define the simulation system completely, we need to describe the geometry of the simulation: how large the cell is, where it resides in space, etc. In this example, we assume that the cell is a two-dimensional circle of radius 5 μm , and the chemoattractant source is positioned approximately 13 μm away from the cell center (*see* [Note 4](#)).
2. To define this geometry, we go to the menu tab in the Bio-model window: File → New → Geometry → Analytic → 2-D. This will open up a “Geometry Editor” ([Fig. 3a](#)).
3. We want an extracellular space large enough to fit the cell and leave room for the chemoattractant source. Click on “Change domain,” and specify the domain size to be a square 15 μm by 15 μm with origin at (0, 0) ([Fig. 3a](#)).
4. Rename subvolume name to “EC,” and leave its value at “1” to represent the EC compartment.
5. Click “Add” to add the IC compartment. Name the subvolume “IC,” and change its value expression to:

$$(X - 6)^2 + (Y - 6)^2 < 25,$$

which is the formula for a circle of with radius 5 μm and centered at (6,6).

6. Save this geometry as “2DLEGI.”

3.4. Linking the Physiological and Geometrical Models

1. So far we have created a physiological model and a geometrical model. Now we link them to form a complete application in the Virtual Cell. Go to the “Applications” panel on the right side of the physiological model, right click on “LEGI” ([Fig. 4a](#)), select “Create Deterministic Application,” and enter the name (e.g., “Needle”) of this new application. Double

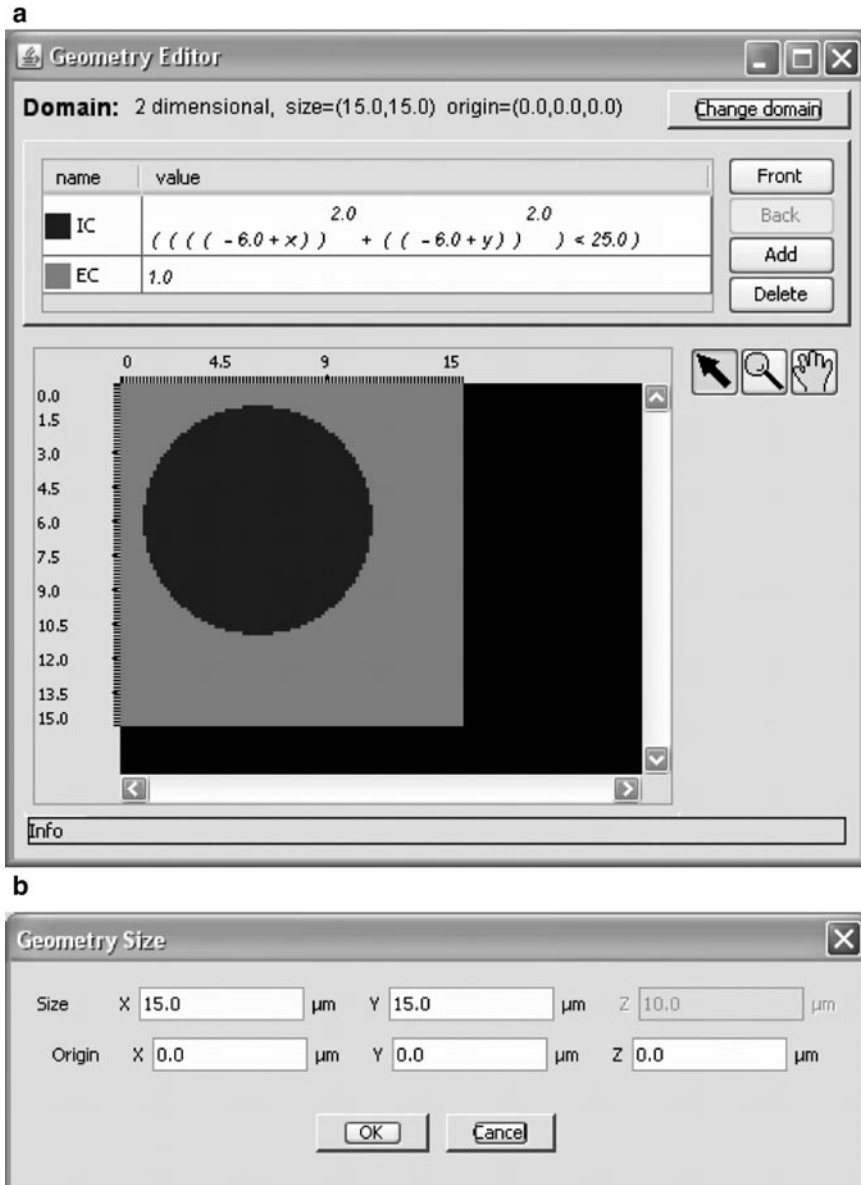


Fig. 3. *Specifying the geometry.* (a) In the “Geometry Editor,” we define the intracellular (IC) and extracellular (EC) geometries. (b) The domain size of the geometry was set in this example to be a square $15 \times 15 \mu\text{m}$ with origin at (0,0).

clicking on “LEGI” to expand it, you see the list of applications associated with this physiological model.

2. We must now link this application to its geometry. Double click on the “Needle” application to open the “Application Editor” (Fig. 4b).
3. Click on “View/Change Geometry” under the “StructureMapping” tab. In the new window, click on the “Change Geometry”

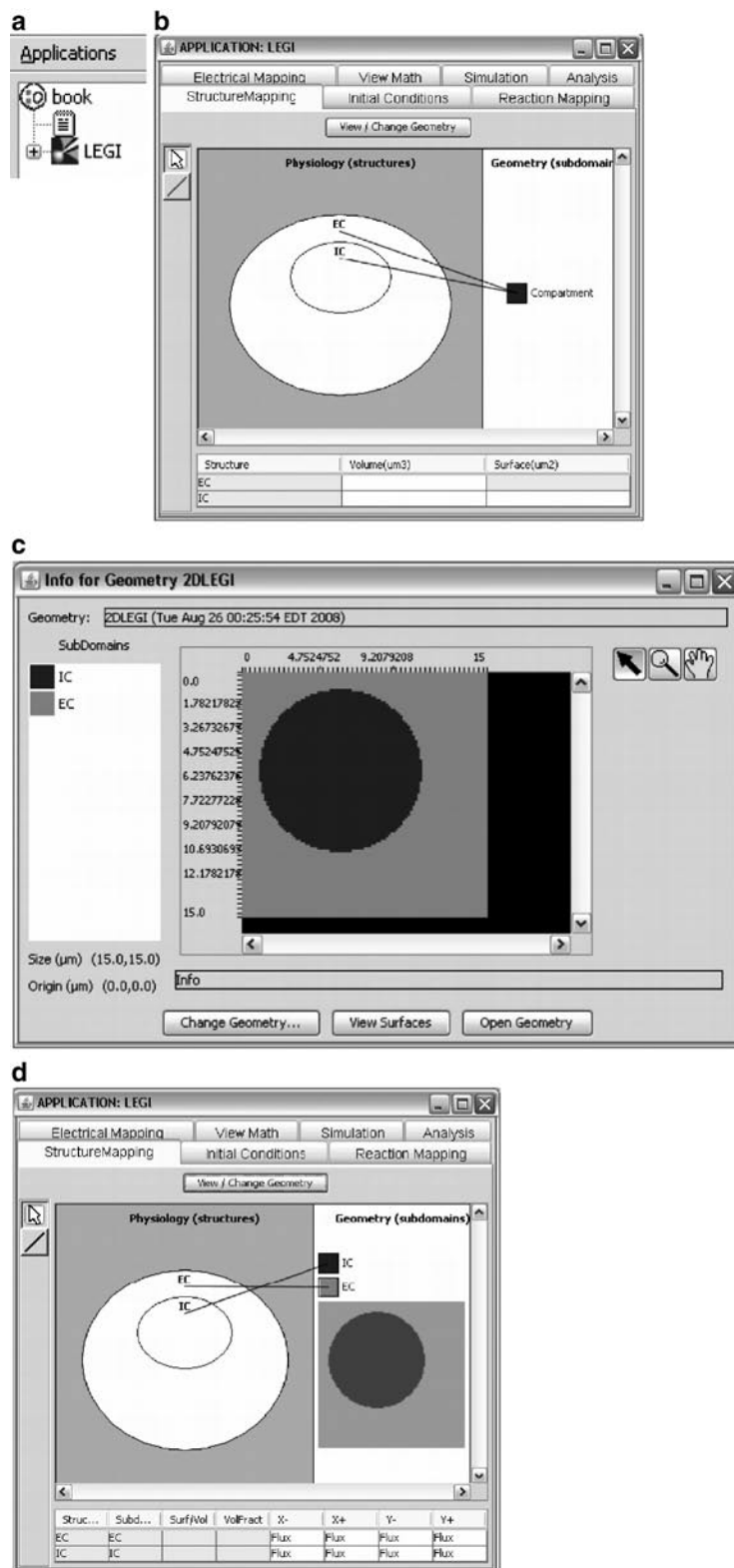


Fig. 4. Linking the physiological and geometrical models. (a) The application panel. (b) When a new application is created, all structures are linked to the same compartment. (c) Specify the geometry to be used for the application. (d) Link physiological structures to geometric compartments.

button, and select the “2DLEGI” geometry you have created. This “Info for Geometry” window now displays the 2DLEGI geometry (**Fig. 4c**). Close this window.

4. Back in the “Application” window, we can link the physiology structures to their appropriate geometries. Click the icon; link “IC” to “IC” and “EC” to “EC” (**Fig. 4d**).

3.5. Specifying Initial Conditions

1. We must specify initial conditions and diffusive properties of each molecular variable in the physiological model. These properties are designed to be part of each application. For each physiological model, many applications can be created; thus, many initial conditions, etc. can be tested (*see Note 5*).
2. In this application, we assume that molecules in the IC and EC are able to diffuse, while molecules in CM are not diffusible. Diffusion coefficients are found in **Table 2** under the “Diffusion” column. Initial concentrations for molecules in all compartments are also found in **Table 2**.
3. Go to the “Initial Conditions” tab in the “Application” window. Click on each molecular species name, and modify the information on the bottom half of the panel to match the information in **Table 2**.
4. The ligand source “L_source” is represented by a fixed concentration (1 μM) at a fixed location (a quarter circle of radius 1 at the bottom right corner of the simulation domain). To define the fixed concentration, check the box under the “Clamped” column (**Fig. 5**). To define the fixed location, use the following expression for the initial concentration:

$$(X - 15)^2 + (Y - 15)^2 < 1$$

5. Save this application.

3.6. Running a Spatial Simulation

1. Click the “Simulation” tab in the “Application” window, and click “New” to define a new simulation. You can change the name of this simulation by double clicking on the simulation name.
2. Select this simulation, and click “Edit.” A new window will open, where you can change the simulation specifications.
3. We can start the simulation at parameters different than those previously defined, or give the simulation a finer spatial resolution by modifying the “Mesh” properties to contain more X and Y elements. For the purpose of this example, we only need to modify information under the “Task” tab. Click the “Task” tab, set “Time Step” to 0.1 s, “End Time” to 500 s, and keep every 10 time samples (**Fig. 6a**). Click OK.

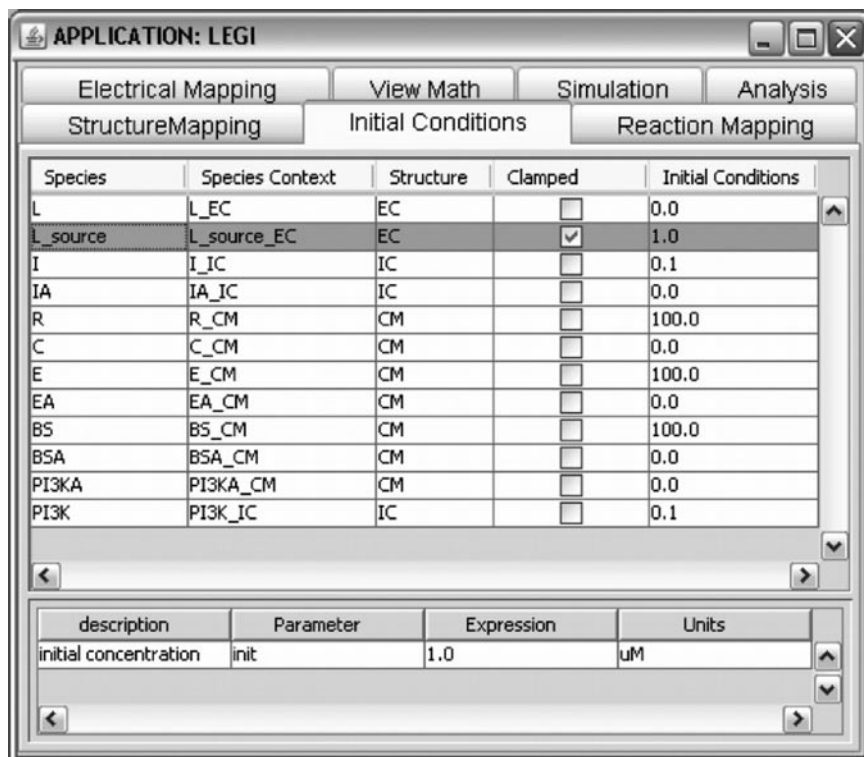
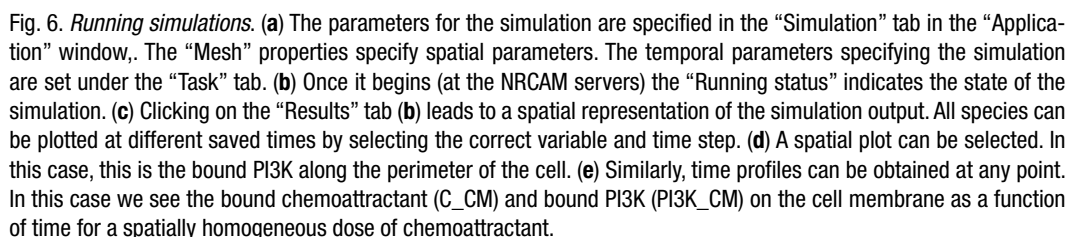


Fig. 5. *Specifying initial conditions.* The initial conditions are specified in this application. Note that, by clicking on the “Clamped” box, the concentration can be fixed throughout the simulation.

4. Back in the “Application” window, select this simulation and click “Run.” The simulation request will be sent to the Virtual Cell server to compute, and “Running status” will indicate “completed” when the simulation is successful (**Fig. 6b**).
5. Click the “Results” button to view simulation results. A new window will open (**Fig. 6c**).
6. In this simulation, we are interested in the spatial distribution of the cell’s response (PI3KA) on the cell membrane. Select “PI3KA_CM” from the variable list. Click on the icon to draw a line around the cell’s circumference. Start at the back of the cell.
7. When the line is drawn, the “Show Spatial Plot” button will become active. Click on it, and a spatial plot of PI3KA will appear (**Fig. 6d**).

3.7. Running a Temporal Simulation

1. We can also simulate the cell’s response to a uniform step function as the chemoattractant stimulus.
2. In the “Applications” panel to the right side of the “Model” window, right click on the application “Needle.” Select “Copy



- As,” then “Deterministic Application.” Name this new application “Uniform,” and open this application to modify.
- Under the “Initial Conditions” tab, check the “Clamped” box corresponding to “L_EC” and change the “Initial Condition” to $0.001 + 0.999 \cdot (t > 200)$.
 - Under the “Reaction Mapping” tab, uncheck the “Enabled” box corresponding to Rx. These last two steps set up the extracellular ligand concentration as a step function going from 0.001 to 1 μM at $t = 200$ s.
 - Repeat **Steps 1–5** in **Subheading 3.5** to set up a simulation. In this simulation, we are interested in the temporal response of the cell’s response (e.g., PI3KA). We can also check that the cell’s receptor occupancy variable C behaves like the input ligand signal. Select “C_CM” from the variable list. Click on the icon, and place a point anywhere along the cell membrane.
 - When the point is placed, the “Show Time Plot” button will become active. Click on it, and a time plot of C will appear. Also plot the time profile for PI3KA. We can confirm that, while the cell’s receptor occupancy displays a step like time profile, the cell’s membrane-bound PI3K (PI3KA) levels display a transient peak before settling back to its prestimulus level (**Fig. 6e**).

4. Notes

- The complete Virtual Cell implementation of the complementary LEGI model ([12](#)) is freely available in the Virtual Cell. To access this model, go to the menu options in the main model window. Click File $\rightarrow$ Open $\leftrightarrow$ Biomodel. Under “Shared Models,” choose “LiuYang” and open the “LEGI” model.
- In the Virtual Cell, the default units are μM for volumetric concentrations and molecules/ μm^2 for membrane-bound species. The spatial dimension is in μm .
- When specifying reactions, make sure to use the variable names used in the Stoichiometry diagram on the top of the “Reaction Kinetics Editor” form.
- Boundary conditions for this simulation assume that there is no flux of protein across the cellular membrane. In situations where the cell and stimulus is symmetric, zero flux boundary conditions can also be used to take advantage of this symmetry. For example, in ([17](#)) a three-dimensional model of the cell used symmetry in the x, y, and z directions was developed. This allowed simulations to run on only one-eighth of the cell, saving both computer time and memory.

5. In setting the initial conditions, one would like to recreate the cell's basal levels. However, as the correct values are rarely known, it is more customary to run the simulation for some time with no (or small) stimulus, and to let the cell equilibrate at this value. In this model we did so by specifying that the stimulus was $0.001 \mu\text{M}$ and letting the cell reach steady state in 200 s, at which point the concentration is increased to $1 \mu\text{M}$.
6. This reaction (#1) is not a true reaction, but rather one way of setting up diffusion of "L" from source "L_source." We can interpret it as the source releasing the chemoattractant (L) at rate k_p with the chemoattractant diffusing through the rest of the extracellular space and degrading at a rate of k_r .

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Chapter 33

Computational Modeling of Signaling Networks for Eukaryotic Chemosensing

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Summary

The task of developing and simulating computational models of signaling networks for eukaryotic chemosensing confronts the modeler with several challenges: (1) The stimuli that initiate the cellular responses one wishes to study are provided by extracellular concentration gradients. This means that the computational model must have a spatially resolved representation of extracellular molecular concentrations. (2) The intracellular responses consist of the generation of intracellular accumulations and/or translocations of signaling molecules, requiring spatially resolved computational representations of the simulated cells. (3) The signaling networks responsible for eukaryotic chemosensing comprise a multitude of components acting as receptors, adaptors, (lipid- and protein-) kinases (including GTPases), (lipid- and protein-) phosphatases, and molecule types used by others for membrane attachment. Models of such signaling networks may become quite complicated, unless one wishes to rely on abstract functional modules with certain input–output characteristics as modeling “shortcuts” replacing subnetworks of biological signaling molecules. In this chapter, we describe how modelers can use a modeling tool (“simmune”) developed to facilitate the design and simulation of detailed computational models of signaling pathways (for eukaryotic chemosensing here), thereby avoiding the technical difficulties typically associated with building and simulating such quantitative models.

Key words: Eukaryotic chemosensing, Computational model, Signaling network, Spatially resolved simulation, Reaction–diffusion system, Modeling software

1. Introduction

The ability of eukaryotic cells to sense small differences in the extracellular concentrations of certain molecules, called chemoattractants, has been challenging mathematical and computational modelers for several decades. With growing amounts of experimental

data elucidating the phenomenon of eukaryotic chemotaxis with increasing detail (1–6) the models changed from purely conceptual (7) to models that investigated possible mechanisms and principles underlying chemosensing (8, 9) toward models that represent attempts to capture as much biological detail as is necessary to obtain directly experimentally testable predictions at the level of basic molecular signaling mechanisms (10–12). Our own efforts to understand how cells of the social amoeba *Dictyostelium discoideum* can translate even shallow extracellular gradients of cAMP into steep intracellular gradients of signaling components such as phosphatidylinositol-(3, 4, 5)P₃ (PIP₃) and PTEN (phosphatase and tensin homolog deleted on chromosome 10) led us to propose a detailed model of interacting proteins and membrane lipids (11, 12). The combination of spatiotemporally resolved experimental analysis of the dynamics of PIP₃ and PTEN concentrations and computational modeling resulted in new insights into the regulation of PI3K activity and predicted the existence of locally controlled negative regulators for this enzyme which were subsequently confirmed experimentally (13). All the signaling mechanisms of the computational model are defined explicitly in terms of molecular interactions, that is, the model does not rely on abstract “amplification” or “translocation” modules that, while reducing the number of hypotheses contained in the model, would also diminish its biological relevance and, importantly, its predictive power. As a consequence of this approach we had to deal with a relatively large number of molecular players and an even much larger number of resulting multimolecular complexes.

Constructing mathematical models of cellular signaling networks “by hand,” meaning: by defining each reaction for molecule complex formation, decay or enzymatic transformation separately is not only very difficult due to the potentially large number of reaction equations. As we will show here, it is not necessary anymore due to the availability of user-friendly computational modeling tools that can be used to investigate the spatiotemporal behavior of signaling network models (11, 14, 15). Based on user-defined simple bimolecular interactions the software package “simmune” generates computational representations of the complete set of multimolecular signaling complexes (up to a user-defined maximum size, i.e., number of molecular components) and their reactions. The software then allows its users to perform simulated experiments, exposing the simulated cells to stimuli either through the application of extracellular molecules or by letting the cells interact with other cells. Detailed information about the intracellular biochemistry of each simulated cell can be obtained and visualized in various ways.

2. Materials

The modeling software we use here has four major components:

1. The core *modeling tool*, “simmod” is used for defining molecule types, specific multimolecular complexes, cell types, the extracellular space and, finally, the basic properties of the simulations to be performed.
2. The cell *morphology modeler* is used to generate and visualize computer representations of cellular structures.
3. The core *simulation tool*, “simmune” is used for performing and interacting with simulated cell biological experiments.
4. The signaling *network browser* is used to visualize the structure and reaction dynamics of signaling networks.

The software runs on Windows XP™ and Vista™, on MacOSX™ and on Linux. It is freely available for noncommercial research. Commercial licenses can be obtained through the NIH Office of Technology Transfer.

3. Methods

3.1. How to Define Properties of Molecule Types, Multimolecular Complexes, Cell Types, Extracellular Space, and Simulations

3.1.1. Defining Simple Molecule Types and Their Alternative States

All the software components can be accessed through the simmod program. After launching simmod select from the *Model* menu item whether to create a new model or load an existing one.

Instead of taking an approach that is only mass-action based and treats cellular signaling pathways essentially as reaction networks consisting of structureless chemicals our modeling approach starts by defining signaling components as molecules with specific functional sites, “binding sites,” used for interactions with other molecules. For the simulations that can currently be performed with our modeling software “simmune,” the basic properties of a molecule, in addition to its binding sites, are its diffusion coefficient and whether it is freely diffusing or constitutively membrane-attached in which case it has to be specified whether the molecule is a trans-membrane molecule or just anchored in one of the leaflets of a membrane. When defining a new molecule, a dialog box will ask for this information (*see Fig. 1a*). After the molecule is defined, its properties can be inspected and edited in the “molecule properties” field of the molecule tab of simmod

(Fig. 1b). To select a molecule to edit its properties double-click its name in the list of “defined molecules” or drag and drop its name into the white region of the “molecule properties” field.

Signaling events propagate reaction networks typically by inducing the modification of molecules, frequently by changing molecular phosphorylation states or conformational properties. The results of such modifications may be interpreted as alternative (states of the) molecules. To define such alternative molecule types select a molecule to edit its properties. Then click “create alternative” (see Note 1).

3.1.2. Defining Molecular Interactions

Drag and drop the two molecule types for which you want to define interactions into the left and right white regions of the “molecule interaction” field of the molecule tab. Then, using the mouse, draw a connection between the molecular binding sites that will mediate the interaction between the two molecule types (Fig. 2a). Click on the small square in the middle of the line to set/edit the interaction rates (association and dissociation rate) (Fig. 2b).

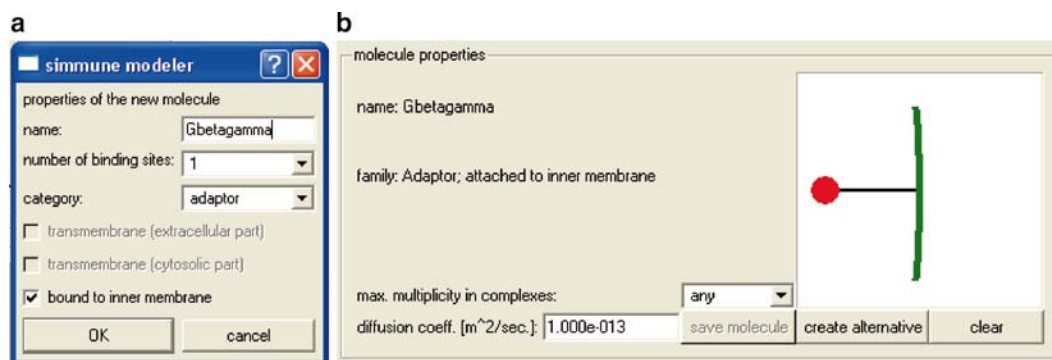


Fig. 1. Defining molecules and their properties. (a) The dialog box for the definition of new molecule types. (b) The molecule properties field of the *simmune modeler*'s molecule tab.

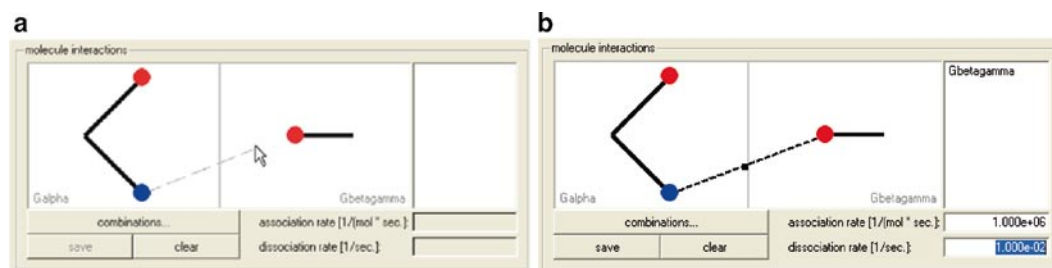


Fig. 2. Molecular interactions. (a) Defining molecular interactions by drawing lines between molecular binding sites. (b) Defining the kinetic rates of molecular interactions.

3.1.3. Defining Transmembrane Receptors

The regions of a trans-membrane receptor located on opposite sides of the embedding membrane typically experience different biochemical milieus. In a *simmune* model, they are therefore first defined as separate molecule types with their individual interaction characteristics (**Fig. 3a**). Then, in a second step they are specified as extra- and intracellular regions of the complete receptor. This is done by dragging and dropping the name of the intracellular domain from the list of defined molecules onto the graphical symbol of the extracellular domain in the “molecule properties” field (*see*(**Fig. 3b, c**).

3.1.4. Defining Molecular Modifications Resulting from Binding- or Debinding- Events

Frequently in signaling pathways, molecules are modified, activated or inactivated, or change their binding behavior, as a result of interactions with other molecules. Among the prototypical examples are the conformational changes many trans-membrane receptors undergo in their cytosolic domains upon ligation of their extracellular domains through their ligands. In models created with *simmune*, such molecular modifications are defined by specifying transitions of molecule types into alternatives states (*see Subheading 3.1.1*). Drag the molecule that will undergo the modification into the *left* white region of the “molecule interactions”

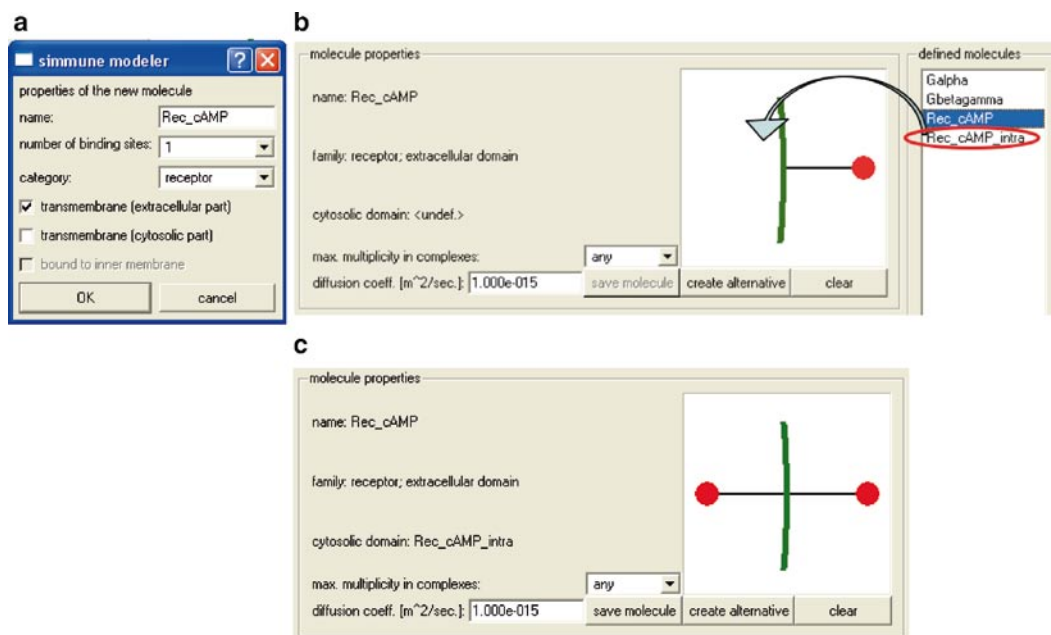


Fig. 3. Trans-membrane molecules. **(a)** Defining a molecule as extracellular domain of a trans-membrane receptor. **(b)** Associating an intracellular receptor domain with the extracellular region by dragging and dropping the name of the intracellular domain onto the graphical symbol of the extracellular domain. **(c)** Once extra- and intracellular domains of a receptor are associated the extracellular region will be displayed together with an intracellular domain.

field and the inducing binding partner into the *right* region. Simmod will indicate the binding possibilities for these molecules as lines connecting the binding sites that mediate the interactions. Double-click the small square of the line associated with the interaction that will result in modifications of the left molecule and click “binding properties” in the dialog box. In the window that will appear specify whether you wish to define a new transformation or remove an existing one. Then, define the characteristics of the transformation: First, whether it is caused by binding or debinding. Second, define what the resulting state (the resulting alternative molecule type) of the transformation is. For trans-membrane receptors, one can specify transformations affecting the cytosolic domains. In that case, both the initial and the resulting state have to be specified (see Fig. 4).

3.1.5. Defining Molecular Complexes

As mentioned in the introduction, simimmune will automatically create the full network of (multi) molecular complexes and their association and dissociation reactions based on the user input of bimolecular interactions (see Subheading 3.1.2–3.1.4). The “complexes” tab of simmod serves to define those complexes that

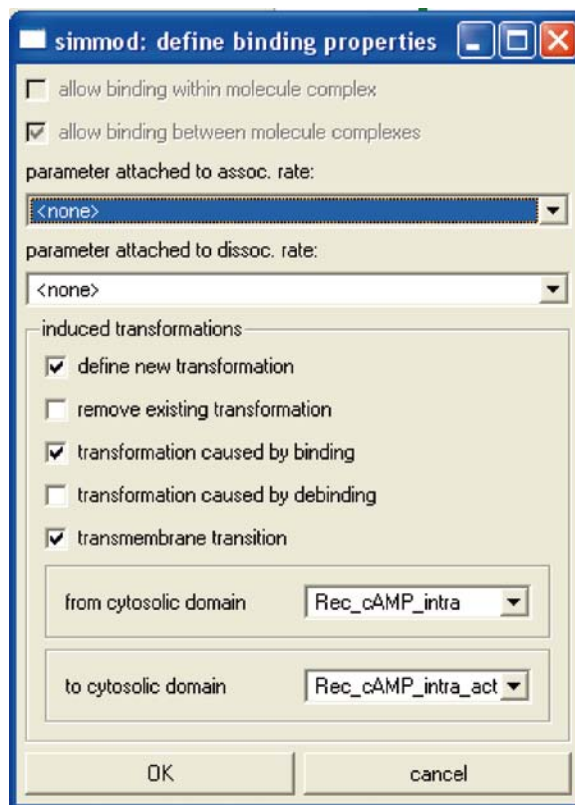


Fig. 4. Defining ligand binding-induced trans-membrane modification of a receptor.

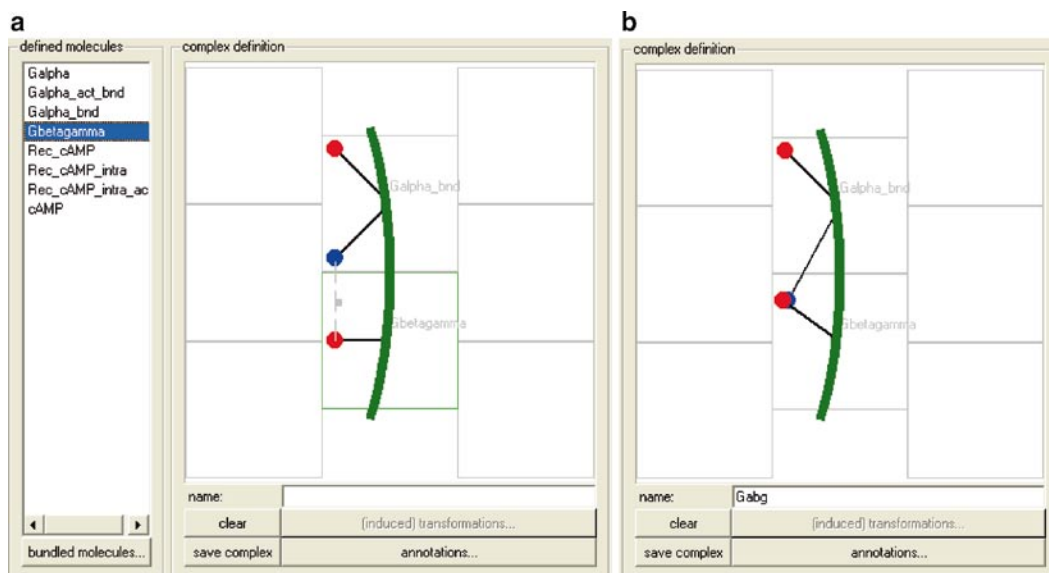


Fig. 5. Molecular complexes. (a) Building molecular complexes by dragging and dropping their molecular components into the “complex definition” field. (b) After double-clicking the lines linking molecular binding sites the sites will be associated.

the modeler wishes to track or manipulate in terms of their intra- or extracellular concentration dynamics or that will be used for the definition of cellular biochemistry (see **Subheading 3.1.9**). In order to define a complex (see **Fig. 5**), drag and drop the names of the molecular components you wish to connect into the white grid of the “complex definition” field (see **Note 2**). Simmod will indicate the binding possibilities for the molecules in the grid. To connect molecular binding sites double-click the small square on their connecting dotted line. When the complex is complete define a name and save it. A graphical representation similar to the one you created in the complex definition field will appear in the list of defined complexes (see **Notes 3–5**).

3.1.6. Defining spontaneous and Enzymatic Transformation for molecular Complexes.

To define spontaneous and enzymatic transformations for a complex double-click its symbol in the list of defined complexes in the complex tab. The complex will appear in the white region of the complex definition field that will now not show a grid (that would be needed for defining the structure of new complexes) (see **Note 6**).

After clicking on the “(induced) transformation” button a dialog box will appear that will allow you to define and remove enzymatic and spontaneous transformations (see **Fig. 6**). Complexes can only be transformed into structurally equivalent complexes consisting of molecule types that are alternatives of the types in the transforming complex. Simmod will only list those

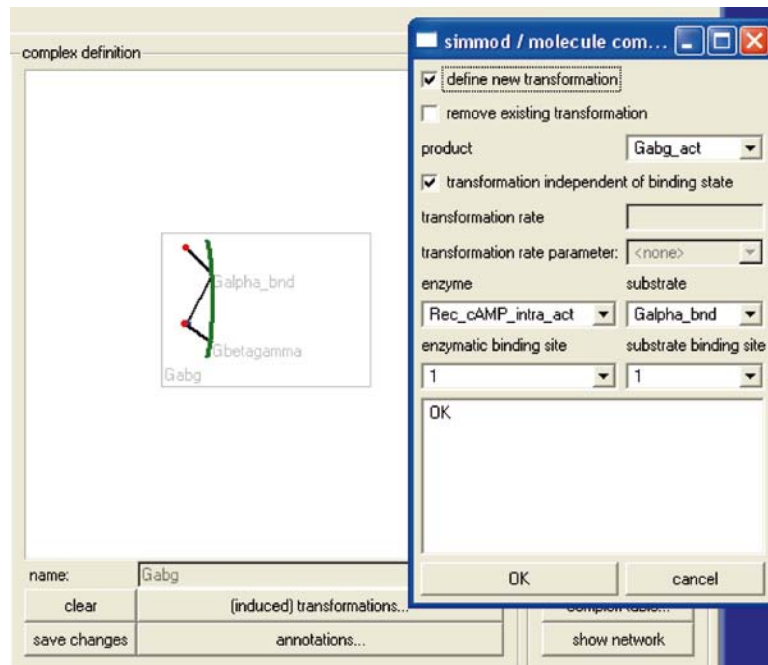


Fig. 6. Defining enzymatically induced transformations of molecular complexes.

as possible products. Checking “transformation independent of binding state” will define the transformation to be performed regardless of whether the complex or the enzyme (for enzymatically induced transformations) is part of larger (unspecified) complexes or not. For enzymatically induced transformations you have to define the molecular details (enzyme molecule, substrate molecule in the complex, binding sites mediating the interaction) of the transformation. Note that by defining the new transformation you have made changes to the properties of the complex that need to be saved. Note that the off-rate between the enzyme and the product molecule will be the enzymatic transformation rate k_{enz} of the enzyme–substrate complex.

3.1.7. Defining Molecule Bundles

Clicking on “bundled molecules” allows you to define placeholders for collections of molecule complexes that contain common components. Click “add bundle,” then specify a name for the bundle. Make sure it is selected (highlighted) and then drag and drop the molecular components that have to be part of a complex in order to make it a member of the bundle into the “contents of selected bundle” field (*see Fig. 7*).

3.1.8. Defining Basic Cellular Properties

A simulation with simmune may contain different cell types. The “cells” tab of simmod allows the modeler to define basic cellular properties for each type, such as lifespan, cell diameter, and the

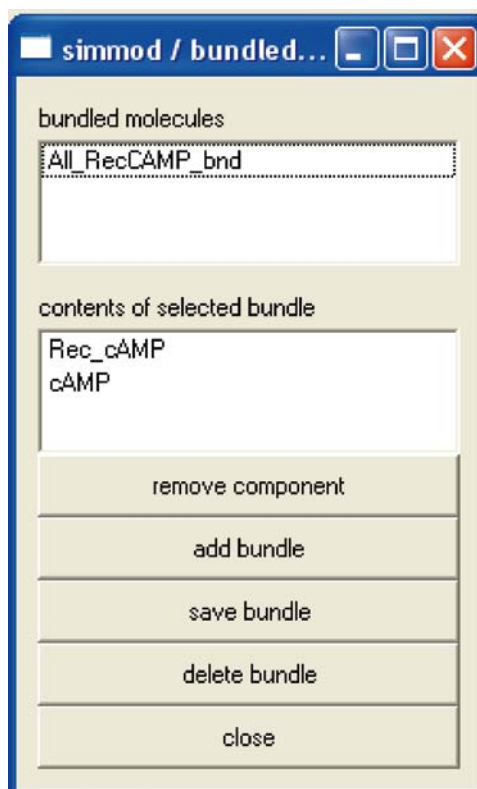


Fig. 7. Defining molecule bundles.

fraction of the cytoplasm that is available for the intracellular biochemistry (the rest could be associated with a nucleus that is not explicitly taken into account). For models of cell types that have a special morphology grid associated with them (*see* [Note 7](#)). To study active cellular movement without the influence of random (quasi-brownian) motion or to simply work with stationary cells check “cell is stationary unless actively migrating.” For an investigation of the chemosensing response in a steady gradient this option should be selected.

3.1.9. Defining Cellular Mechanisms

All fundamental changes (*see* [Note 8](#)) a cell may undergo during its simulated life are initiated through stimulus-response (or condition-action) mechanisms. These mechanisms consist of sets of conditions and actions. If all the conditions are fulfilled the cell will perform all actions. A general treatment of these mechanisms is not within the scope of this chapter. For the purpose of defining the initial biochemistry of cells for the simulation of intracellular signaling networks one defines a dummy condition “INITIALLY” that indicates that the actions will be performed at the beginning of the simulated life of the model cell and a list of actions that create the initial set of molecules (*see* [Fig.8](#)).

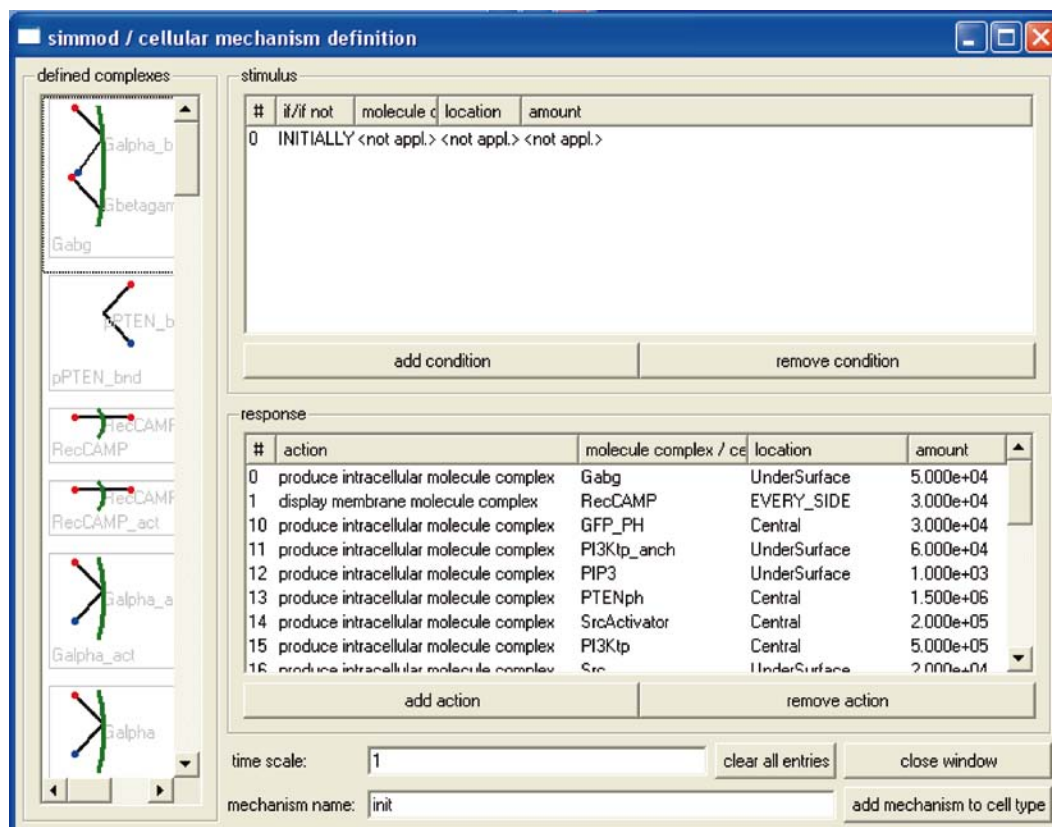


Fig. 8. The mechanism that creates the initial cellular biochemistry.

There are two actions that create intracellular molecules: “produce intracellular molecule complex” for freely diffusing and membrane attached strictly intracellular molecule complexes and “display membrane molecule complex” for trans-membrane complexes or receptors that are on the outer leaflet of the cytoplasmic membrane. After clicking “add action” make sure the new action is highlighted. Then click on the “action” part of the header bar and select the appropriate action. Drag and drop the complex from the list of defined complexes and select location and amount (*see Note 9*). After having defined all actions needed to initialize the cellular biochemistry assign a name to the mechanism and click “add mechanism to cell type.”

3.1.10. Defining the Extracellular Space

The “compartment” tab allows the modeler to specify the dimensions (in units of typical cell diameters of 10 μm) of the extracellular space that will contain the simulated cells and extracellular molecules. For the chemosensing simulations that investigate only the intracellular responses to extracellular gradients it suffices to make the compartment just large enough to hold the gradients

one wishes to apply. For linear gradients the extracellular compartment can be as small as $3 \times 3 \times 3$ units. The cell will then occupy the center position.

3.1.11. Defining the Basic Properties of Simulations

In the “simulations” tab the maximum number of molecular components in the complexes of the simulated reaction networks and the precision of the numerical integration of the dynamics of the biochemistry of the model (*see* [Note 10](#)) can be specified. The external update step defines the time between updates of the visualizations of the simulation (*see* [Note 11](#)). The random generator seed can be varied for stochastic sampling (*see* [Note 12](#)). To prepare a simulation of your model click “new simulation” and specify a name for the simulation. After having set these parameters save the simulation.

3.1.12. Defining Protocols for Automated Simulations of Cell Biological Experiments and Parameter Scans

Protocols in simmune can be used to define at what times during a simulation agents (cells or molecules) should be added to the simulation and what their (initial) location (or for molecules: distribution) should be. Here, we can provide only a short description of how protocols can be used.

To investigate how the behavior of the simulated model is affected by variations in parameter values of rates and amounts (*see* [Note 13](#)) one can define parameter names and then associate these names with parameters of the model. For each parameter name a range of values, a number of steps, and whether the parameter values should be separated by equal distances or factors can be specified. The behavior of the model can be evaluated with the help of concentration displays of molecule bundles that can be selected in the “concentration display” field. When the simulator is started with a simulation definition that includes automatic parameter variation it switches into a noninteractive mode where it displays the selected output time courses organized in a grid according to the parameter values used.

3.2. Fundamentals of a Detailed Model of Eukaryotic Chemosensing and Its Implementation

Here, we describe the fundamental components and mechanisms in the signaling pathway used in our chemosensing model ([11](#)) (*see* [Note 14](#)). For several mechanisms we will give a detailed description of the steps needed to implement them with the modeling software.

1. Binding of the cAMP receptor activates its intracellular domain that then can activate $G\alpha$ as part of the heterotrimeric G-protein complex, that is, when it is bound to $G\beta\gamma$ (“Gbetagamma” in the computer model). To define the receptor portion of this mechanism using the simmune modeler, simmod, define the cAMP receptor as a trans-membrane receptor. Then, define a cytosolic receptor domain. Drag and drop it onto the cAMP receptor symbol in the molecule properties field of the molecules tab of simmod. The receptor and its

cytosolic domain are now connected. Now, define an alternative, “activated,” state of the cytosolic domain molecule. After the ligand, cAMP, has been defined, the binding possibility between the extracellular part of the receptor and cAMP can be defined in the “molecule interactions” field. Using the method described in **Subheading 3.1.4** you can now define the binding-induced activation (and, importantly, a debinding induced return to the basal state) of the receptor.

2. Activated $G\alpha$ releases $G\beta\gamma$, making the two molecule states available to activate downstream pathways. Modeling the properties of $G\alpha$ mentioned so far requires four alternative states of $G\alpha$: a basal state (“Galpha”); a state $G\alpha$ is in when bound to $G\beta\gamma$ (“Galpha_bnd”); an activated, but $G\beta\gamma$ -bound, state (“Galpha_act_bnd”); and, finally, an activated unbound state (“Galpha_act”). Binding of “Galpha” to “Gbetagamma” transforms it into “Galpha_bnd.” Only “Galpha_bnd” has a significant on-rate for binding to the cytosolic domain of the (activated) cAMP receptor. The receptor domain can act enzymatically to transform “Galpha_bnd” into “Galpha_act_bnd” within the complex of $G\alpha$ and $G\beta\gamma$ (*see Subheading 3.1.6* for a description of how to define this). Defining a high off-rate for the binding possibility between “Galpha_act_bnd” and “Gbetagamma” will lead to a rapid dissociation of these components once the modeled $G\alpha$ is activated by the ligated receptor. Note that to obtain the activated unbound state of $G\alpha$ (“Galpha_act”) one needs to define a debinding (from “Gbetagamma”)-induced transformation from “Galpha_act_bnd” to “Galpha_act.”
3. Active $G\alpha$ activates a component that in turn activates a src-like kinase phosphorylating PTEN. Phosphorylated PTEN has a low affinity for its membrane anchor phosphatidylinositol-(4, 5) P_2 (PIP_2). Membrane-localized PTEN has a much higher enzymatic activity on PIP_3 than cytosolic PTEN (**16**). This is modeled by defining two alternative states of PTEN, one of them “PTENbnd” is the result of PTEN binding to PIP_2 and has a higher on-rate for the binding to PIP_3 than the basal state. Dissociation of “PTENbnd” from PIP_2 transforms it back into PTEN.
4. Free $G\beta\gamma$ activates Ras but also induces membrane attachment of RasGAP that can deactivate Ras.
5. Free $G\beta\gamma$ also activates a phosphatase that dephosphorylates phospho-PTEN.
6. Active Ras activates phosphoinositide 3-kinase (PI3K).
7. Active PI3K phosphorylates PIP_2 , thereby producing PIP_3 .
8. In our model, PIP_3 is a membrane anchor for three components: (a) phospho-Gab1, (b) the component activating the

src-like kinase, and (c) SHIP, another component (besides PTEN) that can dephosphorylate PIP_3 .

9. Phospho-Gab1 is part of a feedback mechanism involving the translocation of PTEN. The activity of the src-like kinase that can phosphorylate PTEN (and thereby reduce its membrane affinity) is negatively controlled by a component Csk (17). Csk is brought to the membrane (and hence into proximity of Src) by binding to phosphorylated Paxillin, a membrane-bound component. Phospho-Gab1, which can bind to PIP_3 , recruits SHP2 that dephosphorylates phospho-Paxillin and thereby reduces the availability of membrane anchors for Csk. With reduced levels of Csk, the level of active src-like kinase can increase, leading to increased phosphorylation of PTEN (causing a reduction of membrane-bound PTEN), which in turn allows PIP_3 to increase. This closes the feedback loop.
10. Membrane recruitment of SHIP, in our model, is one of the mechanisms the cell uses to (negatively) control PIP_3 levels once polarization has been achieved.

3.3. Simulating Exposure of *D. discoideum* to Gradients of cAMP

After all molecular interactions, the mechanisms that create the cellular biochemistry, and the extracellular compartment have been defined the “simulations” tab of simmod can be used to start a simulation of the model. After a brief initialization the simulator window of simmune will appear. The simulator offers three views of the simulated compartment: a display of extracellular concentration time courses, a view of the whole compartment and the default view, a 2D slice through the compartment that can visualize extracellular molecule concentration gradients and that therefore will be used here. If the simulation protocol includes the creation of a cell in the compartment, one can start the simulation by simulating a certain amount of time (in our model ~150 s of biological time) to allow the simulated biochemistry of the cell to approach equilibrium. If the protocol does not create a cell click on the “inject” button, select the cell type and the location (“center”) within the compartment and inject the cell. It will automatically be assigned a color code and will be shown as a colored disk in a box (indicating the boundaries of the compartment). Clicking on the disk symbolizing the cell will activate simmune’s “single cell display,” a window offering spatially resolved information about the behavior of the cell’s biochemistry over time. All user-defined complexes and bundles (see **Subheading 3.1.7**) with nonzero concentration will appear in the list of “available complexes.” Double-clicking a complex or bundle name assigns a color code to its concentration time course. The arrangement of the concentration displays (upper, lower, left, right) corresponds to the orientation of the cell shown

in *simune*'s main window "compartment slice view." The peripheral regions each feature a membrane region and a submembrane region showing the concentrations of trans-membrane complexes and intracellular components, respectively (**Fig. 9a, b**) (see **Note 15**). Double-clicking on one of the regions will open a window showing the higher-resolved display of its concentration time courses.

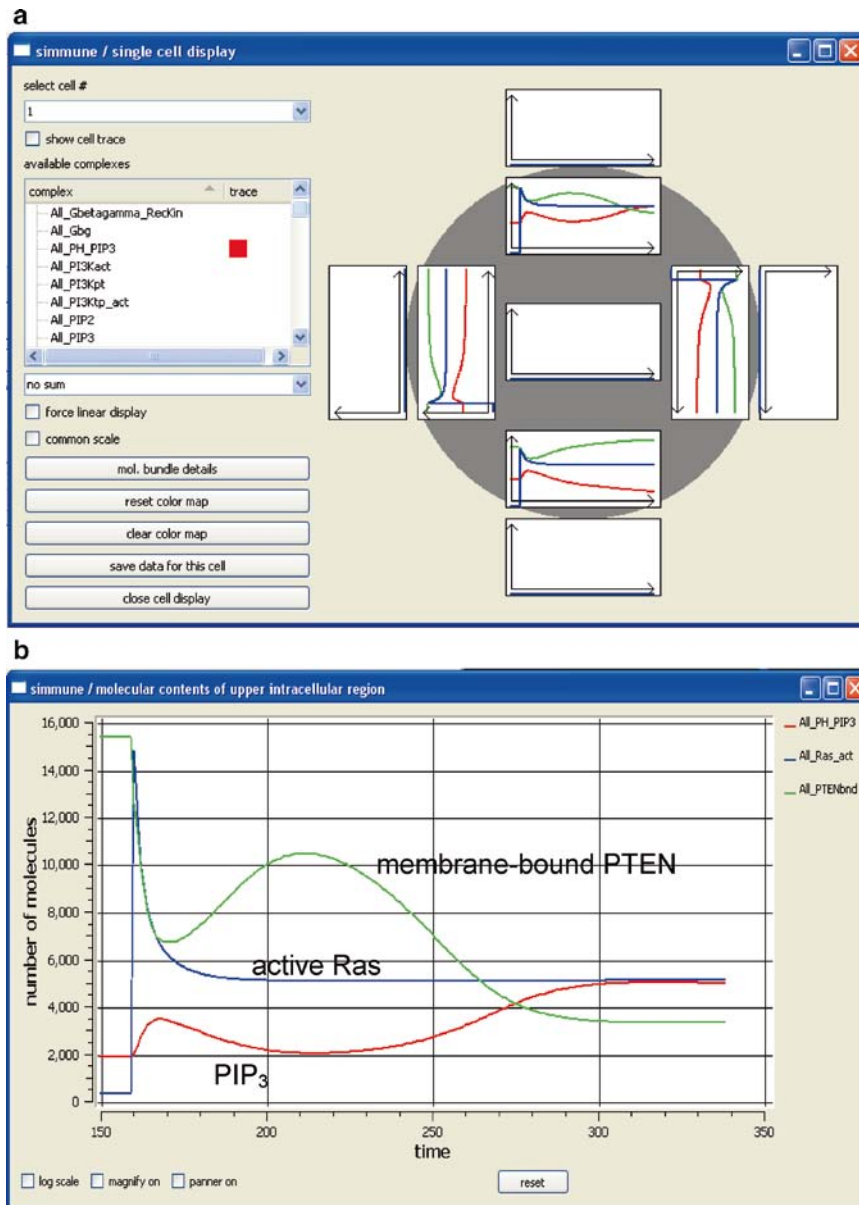


Fig. 9. Inspecting time courses of intracellular concentrations. **(a)** The time courses of membrane-bound PTEN ("PTENbnd" in the text), activated Ras, and of all molecule complexes containing PIP_3 and the reporter PH domain in different regions of the simulated cell in a cAMP gradient. **(b)** A higher detail rendering of the time courses from **(a)** in the region of the cell whose membrane was exposed to higher concentration of cAMP. It shows the characteristic multiphasic and opposing dynamics of PIP_3 and membrane-bound PTEN and the rapid but transient activation of Ras (see **ref. 11**, for more details).

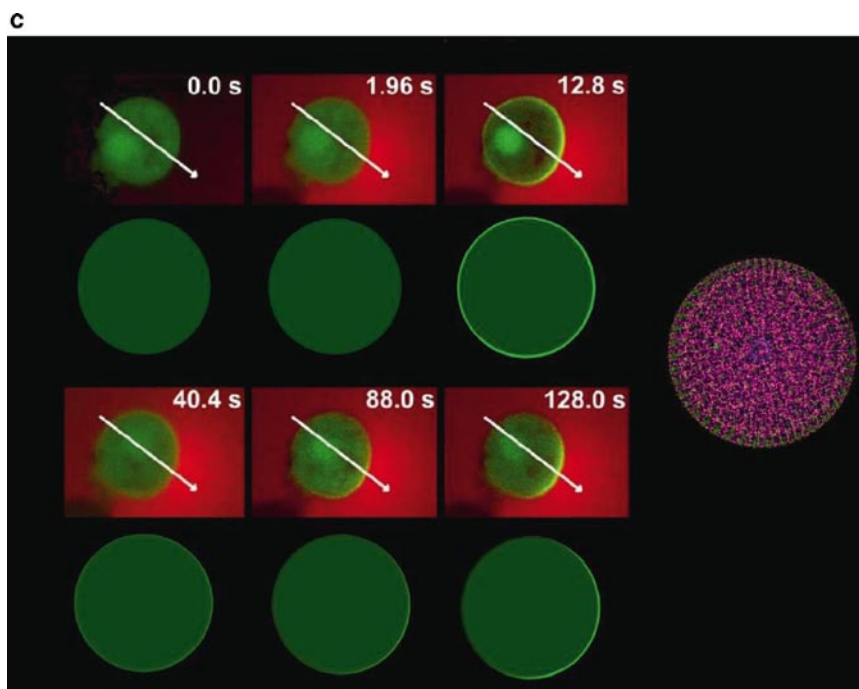


Fig. 9. (continued) **(c)** High-resolution rendering of the simulated exposure of a cell to a gradient of cAMP compared to the real experiment. The images show the transient accumulation of PIP_3 along the entire perimeter of the cell (12.8 s) followed by a cell-wide decay and a second phase of polarization.

To apply an extracellular concentration gradient click “set molecule concentration fields” and select cAMP from the “molecule type” list that will contain all user-defined single-molecule complexes (*see* [Note 16](#)). To set a linear gradient select the direction and define the upper/lower or left/right concentration and click “set concentration field.” The simulation will apply the gradient and create a color-coded (*see* [Fig. 10](#)) display for the compartment slice view.

In [Fig. 9a, b](#), the amount of PIP_3 -associated PH domain over time is shown in red, that of active Ras in blue, and of membrane-bound PTEN in green. [Figure. 9b](#) shows a higher detail rendering of these time courses in the region of the cell whose membrane was exposed to higher concentration of cAMP. [Figure. 9c](#) shows the high-resolution rendering of the simulated experiment compared to the experimental data.

3.4. Analyzing the Behavior of the Simulated Signaling Pathway

3.4.1. Analyzing Biochemical Details

In addition to the concentration time courses the biochemical composition of the simulated cells can be investigated with regard to the question of how the molecules in the cell are distributed over the molecular complexes. After a region of the cell has been selected (double-clicked, *see* [Subheading 3.3](#)) clicking “molecule bundle details” will open a window that lists the fractions of the user-defined molecule bundles occupied by complexes with

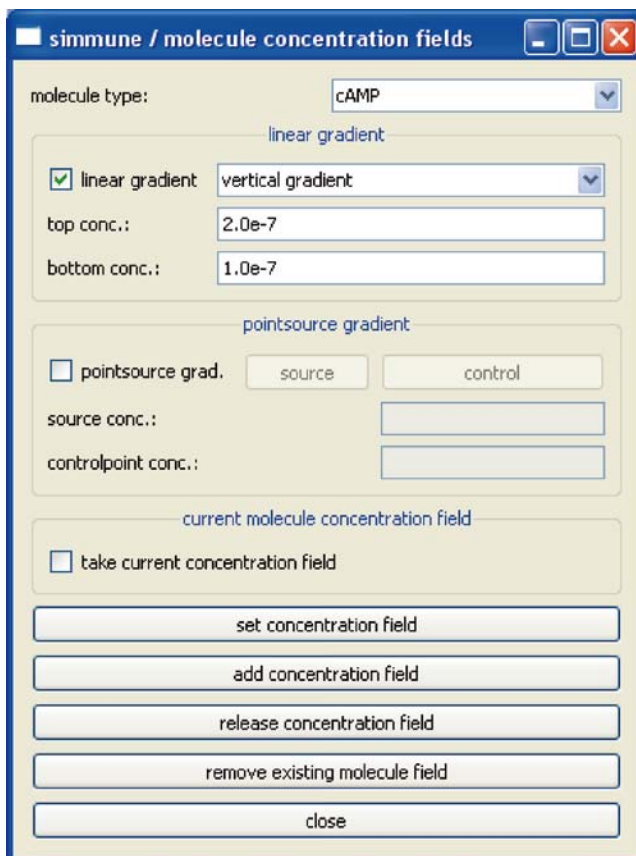


Fig. 10. Selecting the properties of an extracellularly applied gradient.

specific composition (*see* [Note 17](#)). Double-clicking a complex line will assign a color code to this complex composition and show the time course of its concentration in the single-cell displays.

3.4.2. Creating Graphical Representations of the Signaling Network Dynamics

After a cell has been selected for single cell display (*see* [Subheading 3.3](#)) the signaling network dynamics of the cell can be recorded. Click “save data for this cell” then select “signaling network dynamics” as data type and click “start recording.” The data that will be saved allow the network browser that is part of the *simmune* software package to generate network graphs that use color saturation to indicate the changing concentrations of the signaling components and their interaction strengths (reaction flows) ([Fig. 11](#)). Press the play button beneath the pathway display to visualize the signal propagation through the network during the simulated experiment. The cellular region whose concentrations and reaction flows should be visualized can be selected by clicking on one of the “Cellular Compartments” buttons. This allows for comparison of the state of the signaling network in different subcellular locations.

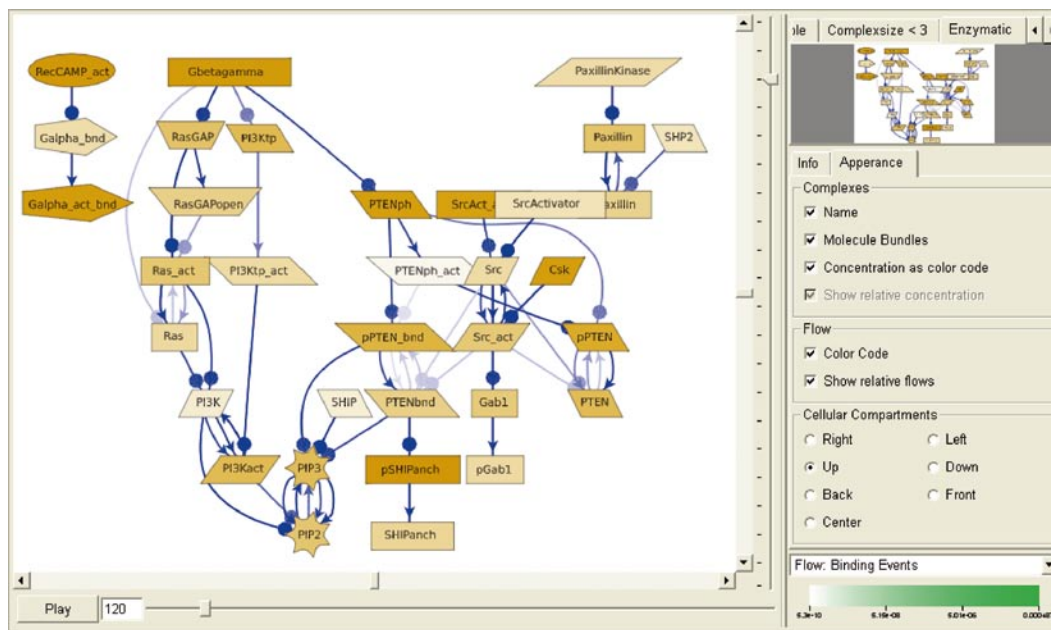


Fig. 11. Snapshot of the automatically created dynamic map of the enzymatic interactions of the chemosensing model from **ref. (11)**. Connections between molecules indicate binding of the enzyme to its substrate by a circular arrow tip pointing to the substrate, the transformation into the product is indicated by a regular arrow tip. The saturation of the lines indicates the momentary enzymatic activity relative to the highest activity of a particular interaction during the course of the recorded simulated experiment. The saturation of the molecule symbols corresponds to their momentary concentration relative to the maximal concentration they reach during the recorded simulation. Moving the time slider below the display one can inspect the changing concentrations and reaction flows.

4. Notes

1. Molecule types and their alternatives are not only identical with regard to the number of binding sites they possess, they also share the same fundamental binding possibilities, meaning that they have the same binding partners, even though typically with different interaction rates. To ensure this correspondence between molecule types and their alternatives simmod treats them not as completely independent types but will rather keep their binding possibilities in sync: whenever a molecule is given additional binding partners the same binding possibilities will be added to all alternatives, however with rather symbolic on- and off-rates of 1.0.
2. When dragging molecules into the complex definition grid of the complexes tab of simmod you will notice that the software will accept drops only for selected positions in the grid, depending on the molecule type being dropped and on the molecules already present in the grid. Membrane-associated molecules can only be positioned into the two central positions.

When the grid is empty, the first molecule has to be put into the upper of the two central positions. These constraints create a certain standard for the complexes but should generally not limit the kinds of complexes that can be defined.

3. When not all the molecules are bound to a common, coherent structure *simmod* will refuse to save the collection of molecules on the grid as a complex.
4. *Simmod* recognizes when a newly defined complex is identical (isomorphic) with regard to the binding structure and the molecular components to an already existing complex and will issue an error message when you attempt to save such a complex.
5. When defining complexes that contain molecule types that undergo transformations as a result of the binding events used to build the complex make sure to use the resulting types in the definition of the complex since you would otherwise have a complex that would never appear in a simulation (its concentration will be constantly zero everywhere). At simulation time, such errors are frequently overlooked by the modeler and may cause some frustration.
6. Transformations of a complex cannot be defined as long as the complex is still being displayed on the complex construction grid. You have to save the complex and then double-click it in the list of defined complexes.
7. *Simmune* can perform simulations with high-resolution representations of cellular morphology. The morphology modeler that is part of the *simmune* software package can transform 3D microscopy data into computational representations of cellular and extracellular structures. These structures can then be associated with cell types and extracellular compartments. A description of this option is, however, beyond the scope of this chapter.
8. Fundamental changes are changes that go beyond directly reaction–diffusion induced changes. The latter do not alter the fundamental molecular composition of the cellular biochemistry while cellular mechanisms may add additional molecules.
9. For strictly intracellular complexes, created with “produce intracellular molecule complex,” the location options are dependent on the type of complex. For membrane-attached complexes, the only option is “UnderSurface.” For freely diffusing complexes, the initial location can be “UnderSurface” or “Central.” Trans-membrane receptors or strictly extracellular components, created with “display membrane molecule complex,” have locations on the membrane that depend on conditions and actions with lower indices in the list. A full discussion of the possibilities is beyond the scope

of this chapter. To populate the surface of the cell homogeneously with receptors select “EVERY_SIDE.” Amount specifies the amount of molecules, not their concentration.

10. For networks with strong feedbacks or other sources of high sensitivity a high precision simulation should be compared to the results of less time-consuming medium precision simulations.
11. The internal update step of the numerical integrators is chosen automatically by the algorithms of the software and is independent of the external update time step.
12. The random generator seed completely determines the sequence of the pseudorandom numbers that will be used for random events in simulations. Depending on the platform the software is used on, the random generator seed may be set globally. This has to be taken into account when running simulation simultaneously.
13. The following parameters can be varied: on- and off-rates for bimolecular reactions, complex transformation rates, amounts in cellular mechanisms.
14. A more detailed description of the signaling network is available in supplementary text 2 and supplementary **Fig.5** of (11).
15. The time course of the displayed concentration starts at the moment the cell is selected for single cell display. The simulation then has to perform several time steps before the concentrations are shown.
16. As mentioned in **Subheading 3.1.5** even *single*-molecular complexes intended for use in cellular mechanisms or for the definition of specific enzymatic transformations or for manipulations performed on running simulations have to be defined as complexes since *simmune*’s simulation interface does not offer access to molecules, only complexes. If cAMP is not listed in the “molecule type” list in the “molecule concentration fields” window you have probably not defined it as a complex.
17. The listing of the complexes in the bundle detail display is unique only up to the biochemical composition of the complexes, not their binding structure.

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Ectothiorhodospira halophila. See *Halorhodospira halophila*

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