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Use of a Microvalve-controlled Microfluidic Device in a Chemotaxis Assay of *Tetrahymena pyriformis* in Response to Amino Acids Released from Bacteria

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Abstract

In this study, we introduce a microfluidic device equipped with pneumatically-actuated valves, generating a linear gradient of chemoeffectors to quantify the chemotactic response of *Tetrahymena pyriformis*, a freshwater ciliate to amino acids excreted from bacteria. The microfluidic device was fabricated from an elastomer, polydimethylsiloxane (PDMS), using multi-layer soft lithography. The components of the device include electronically-controlled pneumatic microvalves, microchannels and microchambers. The linear gradient of the chemoeffectors was established by releasing a chemical from a ciliate-free microchamber into a microchamber containing the ciliate. The ciliate showed chemotactic behaviours by either swimming toward or avoiding the gradient. By counting the number of ciliate residing in each microchamber, we obtained a precise time-response curve. The ciliate in the microfluidic device were sensitive enough to be attracted to 0.1 femtomolar aspartate, suggesting that ciliates as a predator can track down food patches composed of a few bacterial cells. With the use of receptor inhibitors, such as tetraethyl ammonium (TEA) or the grape polyphenol resveratol, we have also demonstrated that the calcium signalling plays an important role in motility and following chemotactic behaviours.

Keywords: Chemotaxis, Ciliate, Microvalve, Microfluidic device, Amino acids

Introduction

Since the first development of microfluidic devices in the early 1990s[1] they have become an important tool for the analysis of chemical and biological processes. When compared to conventional laboratory systems, these devices have the advantages of lower reagent consumption, faster reaction times and greater ease of monitoring. In addition, fluids can be precisely manipulated by controlling channel dimensions and applied forces. Coupling these two features with automated fluid controls makes microfluidic devices excellent platforms for performing chemical and biochemical assays[2,3]. By using a series of crossed intersections and a branched network of channels, chemical gradients spanning up to five orders of magnitude can be generated in a microfluidic device, for example, and thereby enable the assay of chemotactic behaviors of neutrophil and leukemia cells[4]. The term 'chemotaxis' was coined to describe the directional attraction of fern spermatozoid along a gradient of malic or maleic acid salts[5] and ever since has been generalized to the process of cell migration across gradients of various chemo-effectors. Chemotaxis is considered one of the critical cellular processes in the fields of immunology[6], cancer metastasis[7] and developmental biology[8] and recent studies have indicated progress towards improved understanding of the mechanisms linking chemoattractants and their receptors[9].

Tetrahymena pyriformis is an elongated (about 50 μm) unicellular ciliated freshwater protozoan that responds rapidly in response to environmental change and makes it an ideal biological sensor[10,11]. The protozoan plays an important ecological role as predator by feeding on bacteria through the use of chemotactic cues released by the cells[12]. A recent study of ours have demonstrated that *Escherichia coli* produce various amino acids e.g. glutamic acid, aspartate, alanine, glycine, etc[13]. These results hint that ciliates may be able to sense amino acids released from bacteria, and by following them to their source feed upon and destroy them. The traditional method used in studies of chemotactic motility of ciliates is a two-chamber capillary assay[14]. In previous experiments using the assay, 10^{-6} M glycine weakly repelled *T. pyriformis*, whereas 10^{-6} M dipeptide glycine-proline was a strong attractant[14]. Unfortunately, precise control of the chemical gradient of compounds to be test-

ed is not possible with this assay.

Many microfluidic devices have been developed to generate repeatable chemical gradients[4,15]. However, many of the devices were designed to use pressure-driven microflows to generate the gradients, and are thus not suitable for ciliates inclined to follow flow direction[4]. Recently, we reported on our microfluidic platform which was developed to generate a chemical gradient solely by diffusion. Ciliates placed in the device were able to detect as little as 10 pico-molar glycine-proline, which is a five-order lower magnitude than the known sensitivity of ciliates[16]. The device was fabricated using soft lithography[16], which allows incorporation of pneumatic valves[17] to control release of chemo-effectors in a closed microenvironment.

In this report, we report on a quantitative assay for chemotaxis of *T. pyriformis* to the amino acids excreted from *E. coli* by using our previously developed microfluidic device[16]. The microfluidic device was placed on the stage of a conventional stereomicroscope which allowed time-lapse micrographs of ciliates responding to a linear gradient of each amino acid to be obtained.

Results and Discussion

Characterization of Molecular Diffusion between Chambers

The microfluidic device used in this study (Figure 1) contains two inter-connected chambers. The chambers are stand alone or in communication depending on the position of the valve for the interconnecting channel. To characterise diffusion using the device, a fluorescein derivate (MW 691.42)[18] was used for real-time monitoring. The left chamber (LCh) was initially filled with culture medium and then the input and connection valve s were closed, thereafter the right chamber (RCh) was filled with the derivative and closed to the outside environment by valves. After opening the connecting channel, dye from the RCh was allowed to diffuse into the LCh. Images, under UV lighting, were taken every 2 min. The diffusion profile is shown in Figure 2. According to the profile, equilibrium of the dye would be expected after 140 min. To correct for decreasing levels of intensity by quenching due to UV exposure, the fluorescein intensity values were adjusted (data not shown) and fluorescein intensity was shown to decrease by 5.3% over 70 min. Since the velocity of a molecule is inversely proportional to the square root of its mass, the time required to reach the equilibrium for each amino acid can be calculated from the equilibrium time of the dye. Thus, glycine (75.07 MW) and L-glutamate (147.13 MW) were estimated to reach their equilibrium points at 47 and 64 min, respectively.

Chemotactic Response of Ciliates to Amino Acids

Chemotactic responses of ciliates to individual amino acids were analyzed in the range from micro- to zepto-molar levels. As shown in Figure 3, ciliates in the RCh congregated to the LCh that contained 0.1 femto-molar L-aspartic acid. This example demonstrates the superb capability of ciliates to track the bacteria. Depending on the amino acid examined, the sensing limits of the ciliates varied, as delineated in Figure 3B. This broad spectrum of ciliate responses suggests that ciliates can identify and follow food patches composed of bacterial populations at various cell stages and for various conditions. The experiments reported on in this study demonstrate that our microfluidic device is capable of manipulating cells, generating chemical gradients and documenting cell motility.

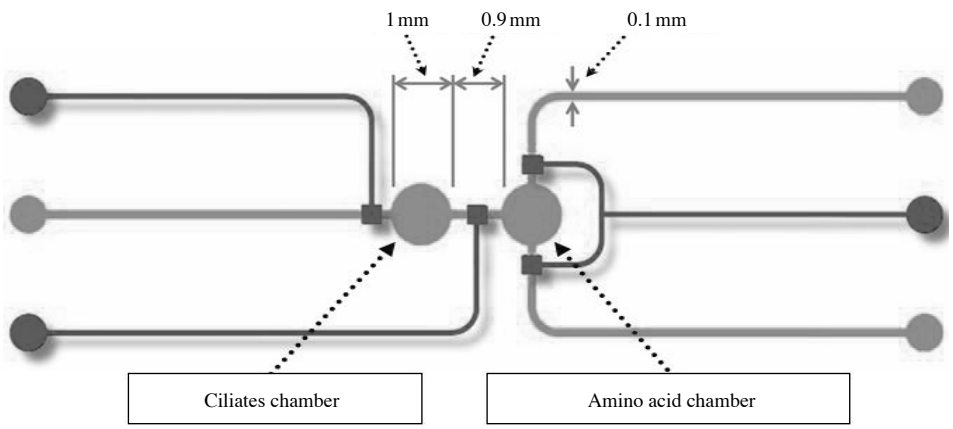

Figure 1. The microfluidic system used as a ciliate sensor. The lower layer represents the fluidic channel, while the upper layer is the pneumatic layer that controls the valves (the squares).

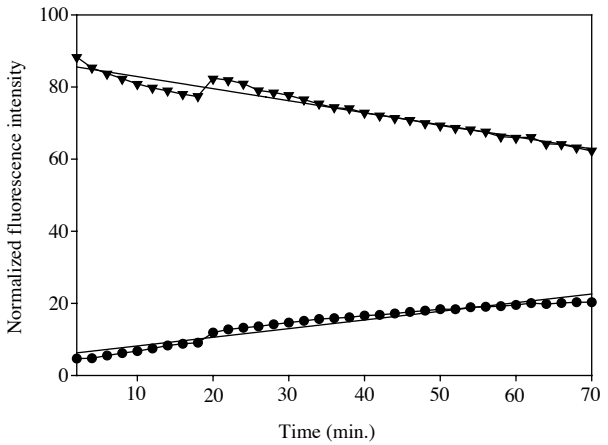

Figure 2. The diffusion of a fluorescein derivative into a microchamber filled with culture medium. The line marked with triangles (▼) is the decrease of dye concentration in the right chamber, while the line marked with circles (●) is the increase of dye concentration in the left chamber.

Perception of Chemo-effectors and Motility

It has been reported that fMLP (n-formyl-methionyl -leucyl-phenylalanine) could be responsible for the chemotaxis of *T. pyriformis*[19] wherein fMLP, a G-protein coupled receptor, activates intracellular calcium release[20]. Calcium is known to regulate various cell functions, especially exocytosis and ciliary movement[20]. By blocking the Ca^{2+} channel cell motility will be impaired[21]. Even though receptors and their signalling pathways have been investigated over many years, there has been no experimental tool to directly prove a correlation between cellular structure and function.

To demonstrate the feasibility of our microfluidic device in the use of such elaborate experiments as calcium signalling, we investigated the effect of known inhibitory compounds on calcium signalling. First, *T. pyriformis* was incubated in 1 mM tetraethyl ammonium (TEA), a fMLP receptor inhibitor for 5 min at 25°C independent of the chip and then the cells were introduced into the chip LCh while 0.1 fM aspartate remained in the RCh. After opening the connection valve, the ciliates incubated in TEA remained in the left chamber. Similar inhibitory effects were observed with ciliates that had been previously incubated with 150 μM trans-resveratol, a grape polyphenol. It is known that trans-resveratol could inhibit fMLP-induced elevations of intracellular Ca^{2+} concentration[22].

Conclusions

Ciliates are subject to follow the direction of subtle hydrodynamic flows and thus it is very difficult to generate a chemical gradient (e.g. in the conventional techniques such as with multichannel micropipettes) without disturbing the protozoa. To analyze their behaviours in response to a chemical gradient in the absence of flow, the ciliates would ideally be expected to migrate according to a chemical gradient generated solely by diffusion. We adopted a well-established microvalve technique in a microfluidic device for this purpose. In our previous report, we showed that the microvalve-controlled microfluidic device is highly suitable for studying the behaviours of ciliates in chemotactically responding to such a dipeptide as glycine-proline. In the present study, we have demonstrated that ciliates, as predators, are sufficiently responsive to sense 0.1 femto-molar aspartate, a level that is excreted from bacteria under aerobic conditions. By this study, we presented the results for a novel microfabricated assay device for the chemotaxis analysis of the ciliated protozoan, *T. pyriformis*.

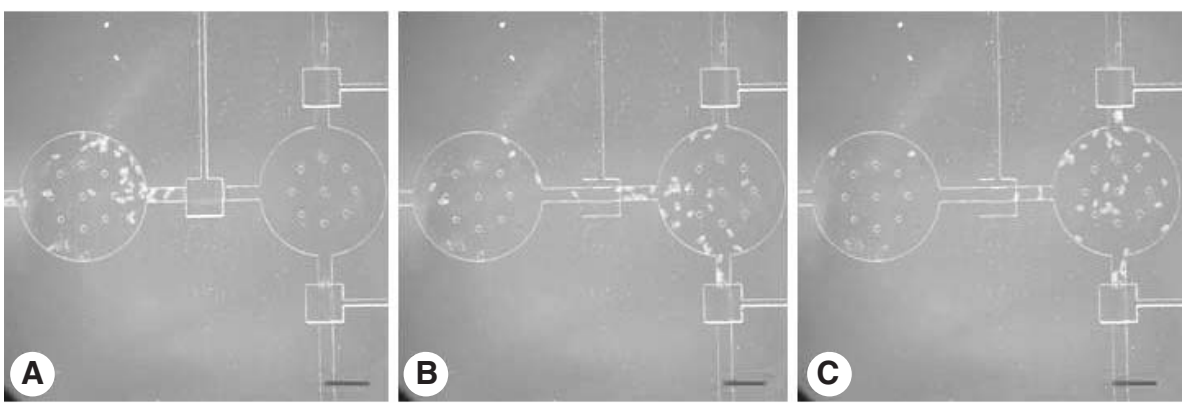

Figure 3. *Tetrahymena pyriformis* chemotaxis to 0.1 fM L-aspartate. (A) Before opening the connecting channel between the two chambers. (B) At 5 min after opening the channel and (C) after 10 min. Bar=300 μm.

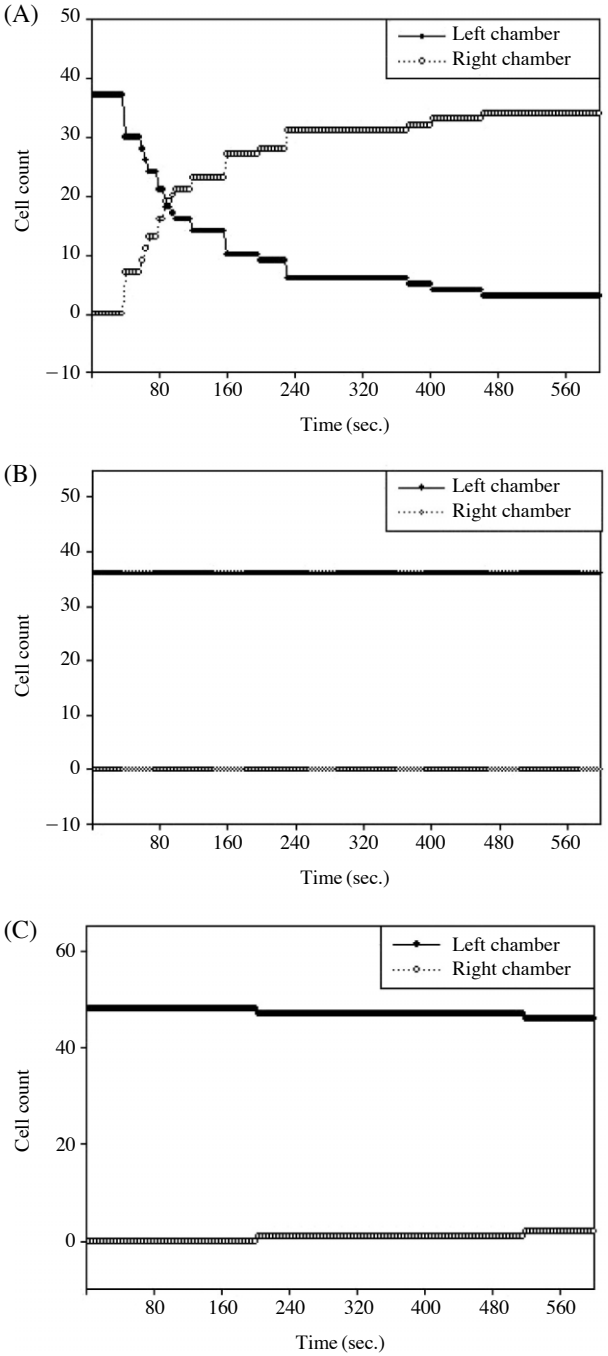

Figure 4. Effect of fMLP receptor inhibitors on chemotactic responses of ciliates to 0.1 fM L-aspartate. (A) Response to 0.1 fM L-aspartate without blockers. (B) Response of ciliates previously incubated with 1 mM tetraethyl ammonium (TEA), a calcium channel blocker, to 0.1 fM L-aspartate. (C) Response of ciliates previously incubated with 150 μM resveratol to 0.1 fM L-aspartate.

The microanalytical device is operated by PDMS microvalves and provides controlled microenvironments for biological research where controllable gradients of chemo-effectors are required. It is possible to observe and record the movement of the cells in real time because of the device transparency. Furthermore, the device was successfully used to quantify the effect of blockers for receptors on the motility of ciliates and in this way allow for the effect of drugs to be measured[21].

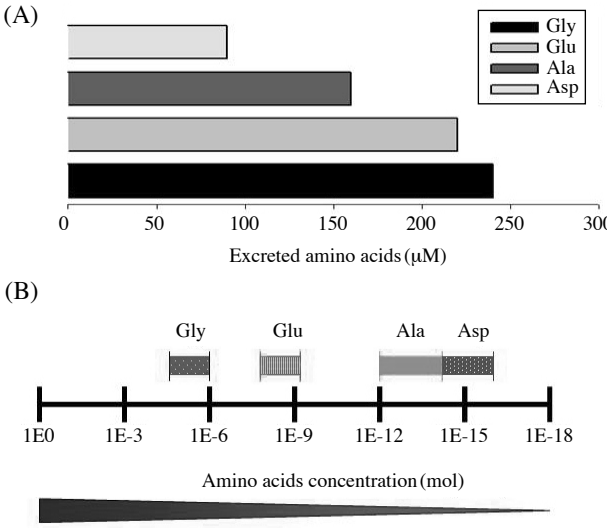

Figure 5. Summary of chemotactic responses of ciliates to the four different amino acids excreted by *E. coli*. (A) Excreted amino acids from *E. coli* (RP437) grown in M9 glycerol medium (reference) after 13 h incubation. (B) The attraction pattern of ciliates to the amino acids at various concentrations.

Materials and Methods

Device Fabrication

The microfluidic chip was made from two layers of PDMS (polydimethylsiloxane, Sylgard 184, Dow-Corning, Cortland, NY, USA), as described previously[16]. In detail, one layer contains fluidic channels, while the second contains pneumatic valves which control the flow on the chip. The top, thicker layer is prepared at a ratio of 1 : 10 (v/v), while the bottom thinner layer is at 1 : 30. Both mixtures were then degassed. "The thick layer was casted onto a master containing the pneumatic valves, while the thin layer was spin-coated at 2,500 rpm for 30 s onto the master which contained the chamber layer." Both layers were then cured for 30 min at 80°C, the holes (thick layer) created using a hand punch (Technical Innovations Inc., Brazoria, Texas, USA), and the layers aligned and brought together. The alignment was done

using a stereo microscope (Dongwon ICT, Daejeon, Korea). The layered structure was again placed in the oven at 80˚C for at least 4 h, where after the holes through the pneumatic layer were punched. The surfaces of PDMS and a microscope slide were simultaneously treated in an O_2 plasma cleaner (Harrick Scientific, Ithaca, NY, USA) at 50 watts for 30 s and the two surfaces were then brought together to form an irreversible bond. A length of tygon tubing (Fisher Scientific International Inc., Hampton, NH, USA) was connect to small hollow metal tubes (New England Small Tubing Company, Litchfield, NH, USA), which in turn were firmly inserted into the punched holes and the tubing connected to a 22 G syringe needle (Korea Vaccine Company, Seoul, Korea).

Ciliates and Reagents

T. pyriformis were obtained from ATCC (Manassas, VA, USA). Cells were grown for 48 h at 25˚C in the axenic ATCC medium 357 containing 5% protease peptone (Difco 0120, BD Co., Franklin Lakes, NJ, USA), tryptone and 0.2% K_2HPO_4 without the addition of antibiotics and without shaking. For chemotactic studies, cells were grown for 12 h in the same medium at 25˚C without shaking. After incubation at 25˚C for 12 h, the cells were grown to early growth phase, to a concentration of $1.1\text{-}1.4 \times 10^5$ cells/mL. Cells were centrifuged at 500 g for 2 min to collect a higher density of cells in a volume of 100 μL and resuspended. Suspensions with the ciliates were applied to a disposable 1 mL syringe (Korea vaccine co., Seoul, Korea). With a syringe attached to tygon tubing and a small metal tube, the ciliates were injected into the microfluidic device. Amino acid solutions were prepared in triple filtered distilled (Human science Co., Hanamsi, Korea) water. Amino acids, tetraethylammonium (TEA) purchased from Sigma Chemicals (St. Louis, MO, USA) and Resveratrol was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). For the control experiment of diffusion, a synthesized diethyl iminodiacetate fluorescein (691.42 MW, ex.=505 nm/em.=510 nm) was used[18].

Experimental Setup and Analysis

The microfluidic device was placed under a stereomicroscope (Nikon, Japan) equipped with a Peltier-cooled CCD camera (Spot diagnostic instruments, Sterling Heights, MI, USA). The pneumatic valves were connected to solenoid valves (Lee Company, Westbrook, CT, USA), which can vent or pressurize the PDMS valves. The solenoid valves were controlled by transistor switches (Comfile Technology, Seoul, Korea) which in turn were operated by a 5V ttl signal from a digital IO card (National Instruments Corporation, Austin, TX, USA). The pressure was set at 30 psi by a mini pressure regulator which was connected to a N_2-gas source. The switching of the valves was controlled by LabVIEW (National Instruments). Chemotaxis of *T. pyriformis* was analyzed by counting cells in the chambers throughout the experiment. All experiments were repeated at least three times, unless otherwise mentioned. For the control experiment of diffusion, a synthesized diethyl iminodiacetate fluorescein dye (691.42 MW) was used. The excitation and emission wavelengths employed were 50 nm and 510 nm, respectively. Fluorescence intensity and the motility of ciliates were measured by Image J program. Data were evaluated by SigmaPlot 2000 program (Systat Software, Inc. San Jose, CA, USA).

Acknowledgements

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