CHAPTER

4

The Facile Generation of Two-Dimensional Stiffness Maps in Durotactic Cell Platforms Through Thickness Projections of Three-Dimensional Submerged Topography

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Abstract

An innovative platform that aims to facilitate studies of how adherent cells migrate in response to rigidity gradients or durotaxis has been developed. Soft polyacrylamide gel–based cell culture scaffolds are used to fabricate flat surfaces containing elasticity gradients through changes in the underlying patterned features. Moreover, this inert gel surface supports long-term cell viability and offers a tunable stiffness. By manipulating the thickness of the gel substrate through the embedded patterns, this system is also capable of directing collective cell patterning.

INTRODUCTION

In recent years, numerous mechanosensing studies have recognized the significance of mechanical cues on cellular response leading to changes in cellular structure and function similar to changes induced by chemical signals. Studies focusing on anchor-dependent cell behavior on surfaces with various elastic moduli reveal that finite mechanical differences could induce cell polarization and direct cell migration, a phenomenon defined as mechanotaxis (Lo, Wang, Dembo, & Wang, 2000). The effects may be further classified as tensotaxis when the movements in adherent cells can be guided by manipulating and creating tension on a flexible substrate. On the other hand, durotaxis is the effect during which the cell migrates preferentially along stiffness gradients on a compliant substrate. A range of cell behaviors including morphogenesis, cell-substratum adhesion, migration, proliferation, and differentiation are affected by the structure and mechanical properties of its microenvironment (Rehfeldt, Engler, Eckhardt, Ahmed, & Discher, 2007). Controlling such biological processes has become a long-standing goal in the development of functional biomaterials. The paucity of good generic testing platforms has posed major challenges in extracting relevant information required to design a functional artificial tissue matrix.

Despite continuous efforts to optimize in vitro testing platforms in mechanosensing studies so that cellular behavior mimics what is found in the in vivo tissue environment, there are still difficulties to overcome. First, the interpretation of results is often complicated by simultaneous changes in both chemical and physical parameters. In particular, studies have used compliant surfaces coated with protein-rich extracellular matrix (ECM) materials such as collagen; fibrin; or a mixture of collagen, laminin, and other proteins including bioactive growth factors forming Matrigel. These ECM protein coatings could form large-scale structures with feature

sizes up to several hundred microns with variable local densities. The resulting topographical cues are known to cause a contact guidance effect where the cells tend to organize along the ECM fibers and use the ECM conduits for migration, leading to measurement bias (Sheetz, Felsenfeld, & Galbraith, 1998). In addition, cellular changes in genotypic traits are reported. The ECM proteins provide abundant integrin receptor binding cues, which are known to trigger an up-regulation of integrin binding subunits that are specific to the ECM (Martin, 1997). These chemical signals from the protein surfaces induce a cascade of complex biochemical signals that again result in measurement bias. The presence of ECM gradients could also direct haptotaxis, another mechanism for directing cell migration (Grinnell, 1986).

Second, the measurements are typically performed on a single migrating cell, as it is believed to help minimize potential cell-to-cell interactions. However, this does not accurately reflect the complex in vivo environment such as that in wound healing, where thousands of cells express collective migratory behavior and migrate toward a targeted area. Finally, most existing platforms are based on skill-demanding multigradient gel preparation or photolithography/nano-fabrication methodologies operating within expensive cleanroom facilities. These high skill and equipment requirements prevent most biological research laboratories from performing patterned rigidity mechanotaxis assays.

Motivated by these complications, a new, simple method has been developed to fabricate flat elastic substrates with a multirigidity landscape and to investigate cellular sensing mechanisms at single and multicellular levels (Kuo, Xian, Brenton, Franze, & Sivaniah, 2012). This work addresses some of the issues raised in the development of biologically inert, rigidity patterned polyacrylamide gel template for use as a model system to study the mechanosensitivity of tissue cells.

4.1 GLASS TEMPLATE TREATMENT

Here we present the method for pretreating the glass template and its top cover glass. The glass template treatment protocol is applied to treat various glass substrates to allow gel to bind strongly to the treated surface once it is cross-linked. The technique has been expanded to include several variations such as the step and the bead system (Fig. 4.1). A method is also presented for treating the top cover glass to allow for easy removal from the cross-linked gel. These treatments can be done in advance on large quantities of templates and top covers, and they can be stored for future usage.

4.1.1 Material

1. Glass coverslip 1, 24 mm $\times$ 24 mm (Academy)
2. Glass coverslip 2, 13 mm diameter No. 0 (Agar Scientific)
3. Grooved glass template, 25 mm $\times$ 25 mm borosilicate glass, 1.8 mm spacing between each groove, 0.2 mm $\times$ 0.2 mm $\times$ 25 mm groove cut (UQG Optics Ltd.)
4. Sodium hydroxide $\geq$98% pellets (NaOH) (Sigma Aldrich)

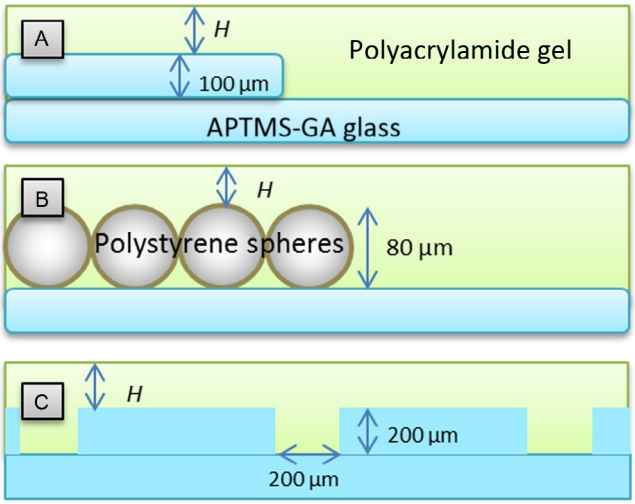

FIGURE 4.1

Illustration of various template cross-sections used to provide rigidity patterned scaffolds. From top to bottom: (A) Step template, (B) bead template, and (C) groove template.

5. RainX Rain Repellent (Halfords)
6. (3-Aminopropyl)trimethoxysilane 97% (APTMS) (Sigma Aldrich)
7. Glutaraldehyde solution, 25 wt.% in H_2O (Sigma Aldrich)
8. UV-cured Adhesive 81 (Northland Products)
9. Duke Standards 4000 Series, 80 µm diameter monodispersed polystyrene spheres (Thermo Scientific)

4.1.2 Equipment

Fume cupboard (Schneider Elektronik)
UV lamp UVLS-28 EL series (UVP)
Ceramic hotplate (Fisher Scientific)

4.1.3 Method

1. To prepare the top glass coverslips, new coverslips, 13 and 24 mm are soaked in RainX solution for a few minutes, the excess is carefully wiped off with tissue paper, and the slide is dried and stored for future use.
2. To prepare the glass template, both the 13 mm coverslips and the 25 mm grooved glass surface are covered with 0.1 M NaOH solution for 1 min.
3. In a fume hood, remove the NaOH solution and add enough APTMS to cover the surface for 3 min.

4. Rinse the glass template surface with distilled water five times to ensure no APTMS remains. If any APTMS remains on the surface, in the subsequent step, it will react with the glutaraldehyde to form an orange precipitate.
5. After cleaning, dry the surface with a compressed air jet.
6. Cover the surface with 0.5% glutaraldehyde solution and allow to sit for 30 min.
7. Rinse the glass template with distilled water and dry the surface with compressed air.
   - The step template is prepared by gluing two 13-mm APTMS–glutaraldehyde-treated glass templates together using UV adhesives.
     a. Apply 1 μl of the UV adhesives to one edge of the 13-mm glass template.
     b. Gently put another 13-mm glass template on top of the adhesive so it covers roughly half of the bottom glass and forms a glass step.
     c. Put the resulting glass step into a UV box for 3 min to cure the adhesive.
   - The bead template is prepared by using monodispersed polystyrene spheres.
     a. Apply one drop of the polystyrene sphere solution on the center of the 13-mm glass template.
     b. Slowly air dry the liquid surface to enable the surface tension of the solvent to pull individual polystyrene spheres together as it evaporates away. Use tweezers to help guide the liquid drop to make sure only a densely packed monolayer array of beads remains in the end.
     c. Briefly treat the bead template with heat at 200 °C for 30 s to partially anneal the beads to the surface.
   - The groove template is prepared by custom preparation of slides. Typically, the blank slides are grooved using a diamond saw machining tool.

4.2 GEL PREPARATION

4.2.1 Material

1. Phosphate buffered saline (PBS) solution without Ca^{2+}/Mg^{2+} L1825 (Biochrom)
2. Acrylamide solution, 40% w/v (BDH)
3. *N*,*N*′-Methylene-bis-acrylamide solution, 2% w/v (Fisher Scientific)
4. Fluorescein *O*,*O*′-dimethacrylate (FDMA), 95% (Sigma Aldrich)
5. Dimethyl sulfoxide (DMSO) (Sigma Aldrich)
6. Ammonium persulphate (APS) (Sigma Aldrich)
7. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) (Sigma Aldrich)
8. Hydrazine hydrate (Sigma Aldrich)
9. Acetic acid (Sigma Aldrich)
10. Poly-D-lysine hydrobromide (PDL) (Fisher Scientific)

4.2.2 Equipment

Fume cupboard (Schneider Elektronik)
Hera Safe KS12 Class II safety cabinet (Thermo Scientific)

Heraeus Fresco 17 centrifuge (Thermo Scientific)
Heraeus Heracell 240 incubator (Thermo Scientific)
Jencos grease-free desiccator (Fisher Scientific)
Pump FB70155 (Fisher Scientific)

4.2.3 Method

Polyacrylamide gel premixes of known bulk shear modulus were prepared according to the procedures developed by Moshayedi et al. (2010). The gel template is prepared on the day of cell deposition.

1. Use the table below to prepare a 500-μl gel premix of the desired mechanical properties. For gel staining, 1% (w/v) fluorescein FDMA in DMSO is added to the gel premix. In some cases, fluorescein may aggregate to form insoluble precipitate in this mixture. To remove the particulates, centrifuge at $10 \times g$ for 3 min and carefully transfer the supernatant to a new Eppendorf tube.

Shear modulus G'	100 Pa	300 Pa	1000 Pa	3.3 kPa	10 kPa	30 kPa
PBS (μl)	392.5	362.5	341	351	295	170
40% Acrylamide (μl)	62.5	62.5	94	94	150	225
2% Bis-Acrylamide (μl)	40	70	60	50	50	100
Stain (FDMA) (μl)	5	5	5	5	5	5

2. Place the gel premix in a vacuum desiccator for 15 min to remove all dissolved gas.
3. Add 1.5 μl TEMED and 5 μl 10% APS to the gel premix, mix the solution, and pipette 20–50 μl of the mixture onto the treated glass template. Avoid any intense mixing, which may introduce air into the solution. The amount of gel premix added will determine the final gel thickness.
4. Gently place the RainX-treated top glass coverslip. If the desired outcome is to produce gel thickness of less than 10 μm, place a small weight on top of the glass coverslip and use tissue paper to wipe away the excess gel premix.
5. Use the remaining gel premix in the tube to determine the extent of cross-link. Depending on the ratio of monomers and cross-linkers, complete cross-linking will take approximately 15 min.
6. Immerse the entire template in PBS for 30 min. Then gently slide the top coverslip off with the help of tweezers.
7. In the hood, treat the gel template with hydrazine hydrate for 3 h.
8. Remove the hydrazine hydrate, and treat the gel template with 5% (v/v) acetic acid in distilled water for 1 h.
9. Move the gel template into a sterile laminar flow cabinet, and wash the gel three times in sterile PBS for 10, 20, and 30 min, respectively.

10. To facilitate cell adhesion, the gels should be immersed in 100-μg/ml PDL solution and left in the incubator for at least 1 h.
11. Wash the gel three times in PBS using the same intervals as before.
12. Soak the gel in tissue culture media for at least 10 min prior to cell deposition.

4.3 CELL DEPOSITION

The method described below is for seeding 3T3 mouse fibroblasts on the gel template. However, the gel template has been successfully tested with the following cell lines: 3T3, HaCaT human keratinocyte, HepG2 human hepatocellular carcinoma, MCF-7 human breast carcinoma, and DLD1 human colorectal adenocarcinoma, and it could potentially be used for all adherent cell types. Nevertheless, cell types of different tissue origin exhibit varying bulk shear modulus preference and elastic moduli range to which they are able to comply and respond by durotaxis. 3T3 fibroblasts were described to durotact on substrates that had relatively wide ranges of mechanical properties (Kidoaki & Matsuda, 2008). On the other hand, durotaxis is also dependent on cellular motility that may result in kinetic differences between cell types exhibiting varying degrees of migratory capacity and may influence the optimal incubation time.

4.3.1 Material

1. 3T3 fibroblasts (ATCC: CCL-92)
2. PBS solution without Ca^{2+} / Mg^{2+} L1825 (Biochrom)
3. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco)
4. Trypsin–Ethylenediaminetetraacetic acid (EDTA), 0.05% (Gibco)
5. Fetal bovine serum (Gibco)
6. Penicillin–streptomycin (PEN/STREP) (Gibco)

4.3.2 Equipment

Motic AE31 Trinocular inverted light microscope (Motic)
Hera Safe KS12 Class II safety cabinet (Thermo Scientific)
Heraeus Heracell 240 incubator (Thermo Scientific)
Centrifuge 5810R (Eppendorf)
Hemocytometer AC1000 (Hawksley)

4.3.3 Method

1. Prepare medium DMEM (450 ml), 10% FBS (50 ml), and 1% PEN/STREP (5 ml). Warm up in water bath for 15 min.
2. Remove flasks from the incubator, and examine the cultures under a microscope. Inspect the cell layer covering the flask surface. The minimal working density of

cultures is 90% confluency. If the flask is at least 90% confluent, the culture is ready for deposition.

3. In the hood, aspirate the media, and wash with PBS once.
4. Add 1 ml Trypsin–EDTA solution to the T-25 flask until the cell layer surface is covered. Store the flask inside the incubator for 5 min. Dislodge the cells from the flask surface by slapping the side of the flask with the heel of your hand 3–5 times. Examine under microscope to make sure all cells are detached.
5. Add 5-ml media to each T-25 Flask. Mix well with Trypsin–EDTA solution to neutralize. Collect fibroblast suspensions into a 15-ml polypropylene centrifuge tube, and centrifuge the contents at 800 rpm for 5 min.
6. Aspirate the spent media, and resuspend pellets in 1-ml fresh media.
7. Count the cell density of the fibroblast suspension using a hemocytometer, and dilute with fresh media to adjust the cell density to 50,000 cells/ml. If different cell types are used, cell density may be adjusted. A strong tendency to form aggregates may be overcome by lowering cell density by a factor of 2 and slightly increasing settling time. In contrast, using higher cell density may lead to difficulties during the image analysis as large cell aggregates and sheets may be formed, biasing image analysis.
8. Transfer individual gel templates to a new culture dish. For each 13-mm gel template, add 300 μl of the fibroblast suspension and 1 ml to each groove template. Allow the cells to settle for 30 min inside the incubator. In the case of cells exhibiting slow adhesion kinetics, settling time may be increased to 60 min.
9. Gently add 5 ml of fresh culture medium to each culture dish, and make sure all the templates are immersed. Store the dishes back in the incubator, and allow the cells to migrate for 16–24 h before imaging.

4.4 STAINING AND IMAGING

4.4.1 Material

1. Cell Tracker Orange CMRA (C34551, Invitrogen)
2. Syto 59 (red fluorescent, S11341, Invitrogen)
3. Leibovit's L15 medium (Sigma Aldrich)

4.4.2 Equipment

Zeiss LSM-510 confocal laser scanning microscope with a 25 × objective (water immersion, NA = 0.8). It is equipped with Argon 488 nm, HeNe 543, 633 nm lasers with its corresponding BP 505-550, LP560, and LP650 filters.
Zeiss LSM Image Browser 4.2.0
ImageJ 1.47 (RSB)

4.4.3 Method

1. Add 5 μm of Syto 59 and 2.5 μm of Cell Tracker Orange to the gel templates, and incubate for 15 min.
2. Aspirate the old medium, and add in sufficient L15 medium to immerse the glass template. This medium allows the cell to remain viable during the imaging session without an environmental chamber.
3. Load the template onto the Zeiss confocal microscope, and start acquiring 3D image stack from random points on the gel. Each image stack will contain the transition zone between the shallow and deep regions of the gel. The z-stack is acquired from the surface of the gel to the surface of the glass template in a step size of 2 μm. This will allow an accurate determination of gel thickness in the subsequent analysis.
4. Analyze the image by counting the number of cells in the shallow N_s and deep N_d regions. The areas A of different regions are measured using the ImageJ software. The gel thickness H is measured from a 3D reconstruction of the z-stacks created using the Zeiss Image Browser.
5. The area normalized fraction of cells in the shallow region is calculated to quantify the level of preferential cell coverage. Cell distribution in the shallow region is calculated as $\phi_{c,s}=(N_s/A_s)/(N_s/A_s+N_d/A_d)$. The higher the value, the greater proportion of cells are found in the shallow region. When the cells remain homogeneously distributed across the whole area, $\phi_{c,s}\approx 50\%$. The average of multiple $\phi_{c,s}$ is plotted against its respective thickness H to determine the critical thickness at which cells are found to migrate.

4.5 RESULTS

The three templates discussed earlier were used to study durotaxis in 3T3 mouse fibroblasts (Kuo et al., 2012). When the fibroblasts were cultured on the step glass system, we found the durotaxis occurred with $H_{step}<15$ μm (Fig. 4.2A). Furthermore, at below this critical depth, there existed an inverse relationship between H_{step} and $\phi_{c,s}$. The maximum amount of cells, between 75% and 90%, were located at the shallow region when the gel was thinnest; $H_{step}=3$ μm (Fig. 4.2B). Surprisingly, changing the bulk shear modulus of the gel from $G'=10$ to 3 kPa or 30 kPa does not affect the $\phi_{c,s}$ ($P>0.05$ for $H_{step}\neq 7$ μm, Analysis of variance (ANOVA).

Similarly, we investigated the fibroblasts cultured on the bead template as a demonstration of the ability to easily incorporate complex alternating stiffness variations in the cell-culture surface. The complementary Atomic force microscopy (AFM) indentation study indicated that the slope of the apparent stiffness gradient was altered by the underlying patterns. The apparent stiffness gradient change was sharper over an abrupt step compared to the smoother gradient change over a bead. It was found that despite the smoother gradient, the critical gel thickness remained consistent, with $H_{bead}<15$ μm (Fig. 4.2C). The inversely correlated association

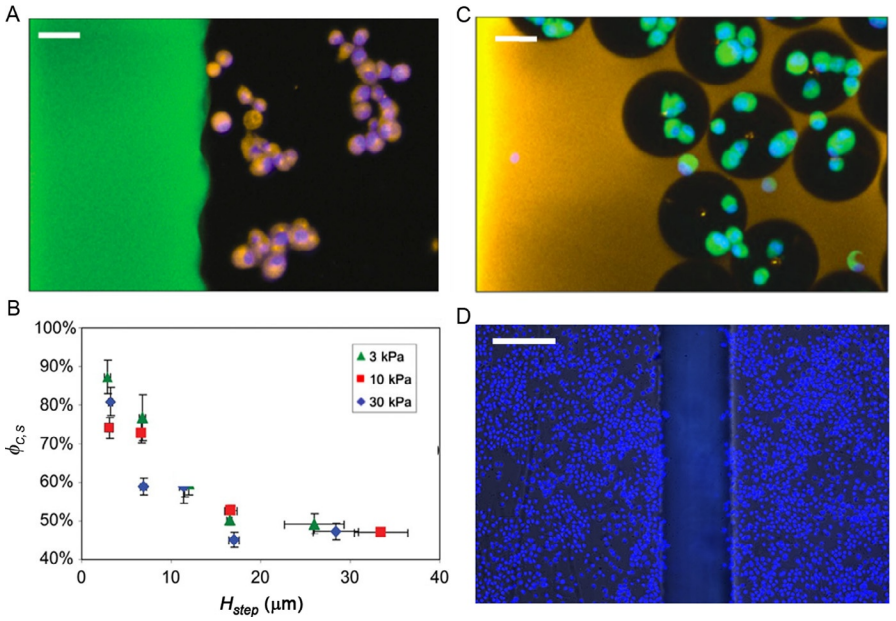

FIGURE 4.2

Cell response to complex mechanical substrates. (A) Confocal laser scanning microscopy (CLSM) images of fibroblasts growing on a step substrate where $H_{step} = 3$ μm and $G' = 10$ kPa. The cells are maximum intensity z-projections of the substrates corresponding to one optical plane. Fibroblasts relocate toward the apparently stiffer region of the substrate. (B) Plot of the area-normalized fraction of cells in the shallow region of the step substrate, $\phi_{c,s}$, as a function of gel thickness and bulk shear modulus G'. Within the investigated range, this critical thickness was largely independent of the bulk shear modulus G' of the hydrogel. Scale bar: 50 μm. (C) CLSM z-projection of fibroblasts grown on a bead substrate where $H_{bead} = 8$ μm and $G' = 10$ kPa. Cells migrated to the area over the beads where apparent stiffness is highest. Scale bar: 50 μm. (D) CLSM *z*-projection of fibroblasts grown on a bead substrate where $H_{groove} = 6$ μm and $G' = 10$ kPa. Cells homogeneously covered the surface of the stiffer region with no cells found over the softer groove region. Scale bar: 200 μm.

Adapted with permission from Kuo et al. (2012).

between H_{bead} and $\phi_{c,s}$ was also unchanged, with approximately 80% of the cells being located on top of the beads for $H_{bead} = 3$ μm. Again, this critical thickness was independent of the bulk shear modulus of the hydrogel ($P > 0.05$ for all H_{bead}, ANOVA).

The groove template represents a slightly different proposition than a single step for the cells since the deep groove presents a finite area of apparently softer gel in between a much larger area of two shallower, stiffer gels to provide a different stiffness profile. Again, the critical gel thickness remained consistent, with

$H_{groove} < 15$ μm (Fig. 4.2D). This system demonstrated that durotaxis could have occurred even at high cell concentrations, creating the potential for massive mechanically guided cell-patterning scaffolds.

4.6 DISCUSSION

The rigidity patterned gel template provides an elegant solution by combining patterning methods and selective chemical modification schemes to control surface-matrix elasticity. It provides a controlled biocompatible surface that can be used to investigate durotactic interactions on the micrometer scales on which cells are organized. As demonstrated, three simple approaches to fabricate complex micrometer patterns provide promising tools to look into cell mechanosensitivity as well for the development of tissue engineering scaffolds. Moreover, the number of the possible diverse patterns to be created at the surface of the glass substrate and the number of the resulting gradient profiles are virtually unlimited. This allows a high flexibility in terms of both gradient shape (discontinuous or continuous) and strength, which in turn determine the range of biologically and medically relevant phenomena that can be studied or modeled in the described system. Significant differences were reported between stiffness gradients encountered by the cells *in vivo* in physiological or pathological conditions. Typically, physiological stiffness gradients occurring during tissue development were found to be shallower (<1 kPa/mm) than those characteristic for pathological events (>10 kPa/mm) such as myocardial infarcts or tumor formation (Tse & Engler, 2011).

It was demonstrated that with appropriate stiffness patterning, it is possible to control the placement of cells in an organized pattern on a chemically homogeneous substrate. Using the durotactic properties of fibroblasts, a strategy to measure the elastic interactions of active cells on rigidity patterned soft gel is proposed. By quantifying the average number of cells that have migrated to the stiffer side with varying gel depth, the level at which cells can feel through elastic tunable gels is discretely determined. In contrast with the previously developed techniques, this technique uses an unbiased charge-based PDL to promote cellular adhesion rather than the specific ECM protein-receptors based solutions. This allows for the unbiased study of the cellular signaling events underlying durotaxis that are also supposed to be dependent on matrix stiffness. This was shown for intracellular linker proteins at focal adhesion sites that underwent force-responsive unfolding to different extents depending on the stiffness of the substrate (Holle & Engler, 2011).

Finally, one significant achievement of this novel procedure is that the rigidity patterned surface requires neither advanced multigradient gel preparation nor access to cleanroom photolithographic patterning facilities. This can enable the study of cell migratory behavior in conventional biology laboratories and can contribute to the development of the next-generation scaffolds suitable for tissue engineering applications.

CONCLUSION

A better understanding of fibroblast migration is essential for the design of materials for regenerative medicine applications. Previous findings suggest that the substrate mechanics response is cell specific. Therefore, future work will apply this technique to study additional cell types involved in wound healing such as vascular endothelial cells and keratinocytes. Subsequently, only when cell-to-substrate and cell-to-cell interaction is understood as one integrated system will its application in tissue engineering material, pharmacology, and genetics be truly rational. As such, there remain many open questions regarding the physical background of mechanosensing, downstream signaling pathways, and the potential mechanisms of cell "memory." Our platform is a useful tool in the ongoing research aiming to answer these questions.

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