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A micropatterning and image processing approach to simplify measurement of cellular traction forces

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Abstract

Quantification of the traction forces that cells apply to their surroundings has been critical to the advancement of our understanding of cancer, development and basic cell biology. This field was made possible through the development of engineered cell culture systems that permit optical measurement of cell-mediated displacements and computational algorithms that allow conversion of these displacements into stresses and forces. Here, we present a novel advancement of traction force microscopy on polyacrylamide (PAA) gels that addresses limitations of existing technologies. Through an indirect patterning technique, we generated PAA gels with fluorescent 1 μm dot markers in a regularized array. This improves existing traction measurements since (i) multiple fields of view can be measured in one experiment without the need for cell removal; (ii) traction vectors are modeled as discrete point forces, and not as a continuous field, using an extremely simple computational algorithm that we have made available online; and (iii) the pattern transfer technique is amenable to any of the published techniques for producing patterns on glass. In the future, this technique will be used for measuring traction forces on complex patterns with multiple, spatially distinct ligands in systems for applying strain to the substrate, and in sandwich cultures that generate quasi-three-dimensional environments for cells.

Keywords

Cell adhesion; ECM (extracellular matrix); Fibronectin; Micropatterning; Traction force microscopy

1. Introduction

A critical process during the execution of virtually every cell function is the generation and application of cell traction forces (CTFs) to the extracellular matrix (ECM) or surrounding cells [1]. Measurement of CTFs has been integral to understanding tumor cell migration and growth [2], the relationship between actin retrograde flow and cell adhesion [3], the co-regulation of cell–cell and cell–matrix adhesion [4–6], and cell structure–function relationships [7,8]. These forces are extremely small, on the order of 1–10 nN. Thus a major technical hurdle in the elucidation of the importance of CTFs to cell function required the development of engineered cell culture environments that could be used to measure these forces with optical microscopy. Increasing the throughput, sensitivity and functionality of these tools has been a major goal in this research field and will permit investigation of increasingly complex cell behaviors. The ability to control the spatial pattern of cell cues, such as growth factors and adhesive sites for cells, is an important determinant of the utility

of these engineered culture environments, but patterning soft materials presents significant technical challenges.

Since CTFs arise in response to cell contraction, initial attempts to measure CTFs were carried out by observing wrinkles made by contracting cells on thin sheets of malleable silicone rubber [9]. More quantitative measurements of CTFs required the development of a cell culture substrate that permitted cell adhesion, but was soft enough to be deflected by micrometers in the presence of nanonewton-scale forces. Mechanically tunable polyacrylamide (PAA) hydrogels are popular substrates for this purpose, although PAA substrates first have to be modified for cell culture due to their inherent non-fouling properties [10,11]. By embedding fluorescent microspheres within a PAA hydrogel, it was found that it was possible to image cell contraction-induced displacement of these fiducial markers and calculate the associated traction forces [12,13]. This technique was recently adapted to measure CTFs for cells embedded in a three-dimensional hydrogel [14].

Advances in micro- and nanotechnology have led to the development of numerous alternative approaches to hydrogels for measuring CTFs, ranging from the use of micropatterned elastomeric substrates to microfabricated cantilever arrays known as microfabricated post-array detectors (mPADs) [15–18]. For example, the use of a cantilever [19] or an array of cantilevers [17] fabricated in a relatively soft material such as polydimethylsiloxane (PDMS) permits quantification of CTFs by measuring the extent of cantilever bending and using simple beam theory to convert displacement to force. The bending stiffness of the beams can be altered by changing their length, diameter or material properties, and more complex substrates with anisotropic rigidity can be fabricated [18]. In addition, fiducial markers can be directly textured onto PDMS surfaces that can be used as cell culture substrates [15].

Although traction force measurements have been greatly improved, each technique retains limitations. With micropost techniques, it is not currently possible to pattern neighboring pillars with different adhesion proteins. In addition, microposts are not easily fabricated to allow external strain application to adherent cells, as has been successfully performed using PAA gels [20]. In the case of PAA hydrogels [21], the lack of a priori knowledge of the position of the embedded beads prior to cell removal prevents real-time measurements from being made. Furthermore, the removal of cells from the substrate to obtain the traction-free positioning makes it difficult to acquire multiple fields of view in a single experiment, thus limiting its throughput. Textured PDMS substrates [15] can be used in a high-throughput manner; however, these surfaces cannot be made with a Young's modulus of less than 12 kPa, which is at the upper bound of physiological stiffness. Finally, both textured PDMS and PAA systems are computationally intense.

In this study, we present a novel image processing approach that addresses some limitations of existing tools for measuring CTFs, including difficulty of pattern fabrication and computational intensity. The ability to reliably micropattern proteins onto the surface of soft hydrogels could dramatically improve upon some limitations of traditional traction measurements. Microcontact printing directly onto soft PAA hydrogels has been described, but this process is challenging and difficult to reproduce [22–24]. Instead, indirect patterning onto a hard surface and transferring onto the hydrogel [22] could potentially render micron-sized pattern markers in defined grids with fluorescently tagged proteins that would make it possible to use the pattern to generate traction force measurements in real time. Our method involves indirectly microcontact printing fluorescently labeled protein dots onto a PAA gel. Cell contraction-induced deformation of the gel is measured based on displacement of the fluorescent pattern, and CTFs can be calculated by using previously established mathematical models [13,25]. By localizing adhesive contacts to a defined area by

patterning proteins to which cells can adhere, the exact points at which the forces are applied and the force-induced displacements can be determined and measured without need for cell removal. Finally, we have made the image processing code widely available through iMechanica blog (www.imechanica.org), thus making this tool available to a broad audience.

2. Materials and methods

2.1. Fibronectin isolation from human plasma and labeling

Fibronectin was isolated from human plasma using a column isolation procedure, as previously demonstrated [26]. Briefly, human plasma in ethylenediaminetetraacetic acid (Valley Biomedical) with 2 mM phenylmethanesulfonylfluoride was first filtered through Sepharose 4B (Sigma), which had been gravity packed overnight and conditioned with 1× phosphate-buffered solution (PBS; MP Biomedicals). The plasma was then passed through a second column that was gravity packed with Gelatin Sepharose (GE Healthcare) and conditioned with 1× PBS. The sample was then washed with 1 M NaCl and 0.5 M urea to remove contaminants. Finally, the fibronectin was eluted with 6 M urea in 1 × PBS. Fibronectin was labeled according to the manufacturer's specifications for protein labeling with AlexaFluor dyes conjugated with succinimidyl ester. AlexaFluor 488 (Invitrogen) was added in excess to fibronectin and allowed to incubate at room temperature for at least 1 h. The fibronectin was then dialyzed using a 10 kDa Slide-a-Lyze Dialysis Cassette (ThermoFisher) for 24 h in 1× PBS to separate free fluorophores from labeled fibronectin.

2.2. Soft lithography

The elastomeric stamp for creating the pattern was fabricated in a multistep process. Briefly, a 1 × 1 cm pattern was created on a chrome-on-glass mask that contained 1.2 μm diameter circular holes spaced with a center-to-center distance of 5 μm. To create the master mold from which the elastomeric molds are cast, Su-8 5 (MicroChem) negative photoresist was spun onto a silicon wafer (University Wafers), which had been cleaned with Piranha solution. The spinning, UV exposure and heating steps suggested by the manufacturer were followed to create the desired pattern with a height of 5 μm. PDMS (Sylgard) was mixed in a 1:10 ratio of elastomer to base and degassed for 30 min before pouring onto the mold. After pouring, it was degassed for another 15 min to further remove any created bubbles, then baked at 80 °C for at least 2 h before being removed from the mold.

2.3. Surface patterning

Fluorescently labeled fibronectin was patterned onto a thin PAA gel layer (90 μm thick) using an indirect patterning technique (Fig. 1). First, fibronectin at a concentration of 50 μg ml⁻¹ in PBS was adsorbed onto the surface of a plasma-treated PDMS stamp for 30 min. Excess fluid was then removed from the stamp by gently blowing it dry with a filtered air gun. The stamp was brought into contact with a glass coverslip, which had been cleaned by sonication in ethanol and water before being plasma treated. Light finger pressure was applied at the beginning of the process to enhance contact with the glass. After 10 min, the stamp was removed and discarded, as it was found to significantly degrade with each successive patterning.

Next, the pattern was transferred from the coverslip to a PAA gel. A second glass coverslip, serving as the base for the gel, was treated with a 5 vol.% solution of 3-aminopropyltrimethoxysilane in distilled water for approximately 15 min. The coverslip was vigorously washed several times with distilled water and then immersed in a solution of 0.5 vol.% glutaraldehyde in distilled water for 30 min and subsequently rinsed with distilled

water before being dried with a filtered air gun. This coverslip forms covalent bonds with the PAA gel. The coverslips can then be stored under vacuum for up to 3 weeks prior to use.

The PAA formulation used in the experiment was a solution of 0.07% bis-acrylamide and 10% acrylamide (Fisher Scientific). The bis-acrylamide and acrylamide were first combined and allowed to rest for 15 min to degas. Next, 10 μ l of tetramethylethylenediamine (Fisher Scientific) was added to the 5 ml of PAA solution. Since acrylic acid *N*-hydroxysuccinimide (NHS)-ester (Sigma-Aldrich) would be neutralized due to the basic nature of the solution, 2 M HCl was added until the solution reached a neutral pH of approximately 7–8. It is important to add the NHS-ester (50 μ l of 1 mg ml⁻¹ for a 5 ml gel precursor solution) after this step so that it does not become neutralized by the basic pH of the PAA solution. Finally, 25 μ l of a 100mg ml⁻¹ solution of aminopropylsilane (Fisher Scientific) was added to the 5 ml gel precursor solution to initiate the polymerization process. The solution is allowed to polymerize for 90 min between the patterned coverslip and the glutaraldehyde-treated coverslip. NHS forms covalent bonds with amino groups on the patterned protein so that the pattern transfers to the PAA gel after removal of the upper coverslip. To passivate any remaining active NHS groups on the gel, a solution of 4% bovine serum albumen (Fisher Scientific) is incubated with the gel for 1 h.

2.4. Mechanical properties of hydrogels

The elastic moduli of the gel were acquired from the material testing of the bulk properties of the hydrogels. The complex shear modulus (G^*) was measured by parallel plate rheometry, while the Poisson's ratio (ν) was determined by tensile testing. Samples for shear measurements with a thickness of about 1 mm were prepared and allowed to swell in PBS overnight at 4 °C. An AR2000 Rheometer (TA Instruments) with the Rheology Advantage software suite was used to analyze the data acquired. The rheometer rotating plate was a 40 mm diameter aluminum disk. First, a test gel was analyzed to determine the linearity of its material response. Using a constant frequency of 1 Hz at a temperature of 37 °C, the sinusoidal stress was applied over a range from 0.1 to 1000 Pa and the corresponding shear strain was measured. The gel exhibited a linear stress–strain behavior in this range. Next, using a sinusoidal stress of 0.4 kPa amplitude, a frequency sweep was conducted from 0.01 to 10 Hz and G^* was obtained as a complex stress to strain ratio. The real part of G^* represents the elastic (storage) shear modulus (G), whereas the imaginary part of G^* is the viscous (loss) modulus. It was found that G was constant, with an average value of 1.261 ± 0.045 kPa ($n = 4$). Over the tested frequency range, the phase angle was $0.03 \pm 2.31^\circ$, indicating that the material was linearly elastic. To determine ν , 3.3×7.11 cm gel samples of a uniform thickness of 1 mm were stretched uniaxially and changes in the axial and transverse directions were measured for increasing uniaxial force. Assuming material isotropy, ν was calculated as the negative ratio of the transverse to the axial strain. It was found that the average $\nu = 0.445 \pm 0.042$ ($n = 4$).

2.5. Cell culture

NIH 3T3 fibroblasts were acquired from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% newborn calf serum and 1% penicillin and streptomycin. During time-lapse experiments, cells were kept in an environmental hood on the microscope that maintained 100% humidity, 37 °C and 5% CO₂. Cells were seeded onto the patterned PAA gels at 400 cells cm⁻² and cultured overnight before image acquisition or initiation of a time-lapse experiment.

2.6. Image capture and processing

The images of the fluorescently labeled fibronectin patterns were taken using an Olympus IX81 microscope with either a $\times 40$ water (1.15 NA) or $\times 60$ oil (1.42 NA) objective and a

Hamamatsu Orca R2 camera controlled with Metamorph software. A custom script written in MATLAB (The Mathworks, Cambridge, MA) was used to perform tracking and analysis of the images. The script is available on www.iMechanica.org. First, the fluorescent image of the pattern in TIF format is read into MATLAB. The fluorescent points are then located using a MATLAB version of IDL particle tracking, developed by Blair and Dufresne (<http://physics.georgetown.edu/matlab/>). A bandpass filter is applied to the image to remove the background. Next, the image is thresholded and the program identifies the geometric center of each dot based on its shape. The user is then asked, through a graphical user interface, to identify the four corner positions of a rectangle within which dot displacements and traction forces will be quantified. The program then determines the centroidal displacement vector $\mathbf{u}$ of each dot from its assumed traction-free position on the grid pattern, and the corresponding traction force vector $\mathbf{T}$ is calculated assuming a uniform tangential traction stress distribution over a circular area on an isotropic elastic half space [25]

$$\mathbf{T} = \frac{2\pi G a \mathbf{u}}{2 - \nu} \quad (1)$$

where a is the radius of the patterned dot. A salient assumption is that the displacement of an individual dot results primarily from the force applied at the dot and not from forces acting at the neighboring dots. This seems reasonable, considering that the center-to-center distance between the dots is ~ 5 dot diameters, while the displacement decreases inversely with the radial distance from the center [25].

3. Results

3.1. Surface patterning

First, we sought to improve the measurement of CTFs by generating a pattern of cell adhesive proteins on the surface of a PAA gel. Although direct microcontact printing onto the surface of PAA gels has been reported in the literature [27], we were not able to reliably generate patterns of dots with this technique (data not shown). Furthermore, sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH) is often used as a linking chemistry to covalently attach protein to the surface of the PAA gel after gelation (see e.g. [12,22,28]), but we found that sulfo-SANPAH is difficult to reliably transfer patterns over large surface areas (data not shown). Instead, we opted to use NHS-ester to covalently bridge the PAA polymer with amine-containing proteins [29,30]. First, fluorescently labeled fibronectin was adsorbed onto a PDMS stamp (Fig. 1A). The stamp was used to microcontact print an array of dots onto glass (Fig. 1B), which was then used as part of a coverslip sandwich within which PAA pre-polymer and NHS solution was allowed to polymerize (Fig. 1C). Removal of the upper coverslip resulted in transfer of the pattern to the PAA gel (Fig. 1D), thus rendering it suitable for blockage of non-specific adhesion sites with BSA and subsequent cell culture (Fig. 1E). Low-magnification images demonstrate that microcontact printing fluorescently labeled fibronectin onto glass resulted in pattern transfer over very large surface areas (Fig. 1F); areas as large as 1 cm^2 could be easily patterned. Similarly, the pattern could be transferred to the PAA gel surface with high fidelity (Fig. 1G). The average diameter of microprinted dots was $\sim 1.2 \text{ }\mu\text{m}$, so we used this value in the subsequent calculations of CTFs.

Since the PAA gel is functionalized with NHS-ester, which may covalently attach to cell adhesive proteins in serum or directly to cells, we next tested whether incubation of the gel after polymerization with albumin is sufficient to quench remaining NHS groups and thus inhibit cell adhesion on unpatterned regions of the gel. A circular region of fluorescent fibronectin was first adsorbed onto a coverslip that was then used to transfer the pattern onto

a PAA gel. Fluorescence and brightfield images (Fig. 2A and B, respectively) indicate that, even after 24 h of cell culture, cells are limited to the fibronectin-patterned region of the cell (Fig. 2B).

3.2. Image processing and calculation of CTFs

We developed a custom script in MATLAB that can be used to convert the fluorescence images of the fiducial dot markers into displacement and traction vectors. First, we used this program to analyze the inherent error associated with our technique for patterning the gel, since patterning errors may lead to apparent dot displacements even in the absence of applied force. A sample image of fluorescent dots on a $G = 1.261$ kPa PAA gel is shown, with apparent dot displacement vectors added for each dot that were determined from the analysis program (Fig. 3A). The cumulative distribution of all 450 displacement vectors analyzed in this field of view is shown in Fig. 3B, where the average displacement was 0.10 ± 0.05 μm . From these data, we can determine that a threshold displacement value of 0.3225 μm , or 3 pixel widths, and the corresponding traction force of 0.98 nN with this gel stiffness, would eliminate close to 100% of background vectors. Consequently, we adopted this value and assigned all displacements > 0.3225 μm to a value of 0 in subsequent cell studies (otherwise, the perceived strain energy of a dot pattern in the absence of cells would have a non-zero value). Because of this thresholding, cell contraction-induced displacements of > 0.3225 μm would incorrectly be perceived as a false positive and eliminated, which represents a fundamental limitation of our system.

Next, we used our displacement analysis program to calculate the CTFs applied by an NIH 3T3 fibroblast cell to a $G = 1.261$ kPa PAA gel. The fluorescence image of the dots underneath the cell clearly indicates that CTFs displaced the dots (Fig. 4A and B). The displacement vectors, shown to scale (Fig. 4C), had magnitudes that ranged from 0 to 2.6 μm . The cumulative distribution plot of displacements for all dots in this field of view indicates a clear distinction between background displacement vectors that were less than ~ 0.3 μm , which is consistent with the error analysis in Fig. 3, and dots displaced due to cell traction forces (Fig. 4D). All displacements above 0.3225 μm were converted to traction forces using Eq. (1) (Fig. 4E and F). These data indicate that the maximum force applied by the cell was ~ 8.5 nN. Importantly, this maximum force did not cause displacements greater than the above background displacement of the neighboring dots lying beyond the cell border (Fig. 4F). This, in turn, indicates that the displacements of individual dots result primarily from the forces applied at the dots and not from forces acting at the neighboring dots, which justifies the application of Eq. (1).

As an additional control to quantify the accuracy of this technique, we balanced the forces in Fig. 4E. Balance of forces demands that all traction vectors sum up to 0. After application of the 3 pixel displacement threshold, we summed all force vectors and divided the magnitude of this remaining “unbalanced” vector by the sum of the magnitudes of all above-background force vectors. The magnitude of the unbalanced vector constituted 8.4% of the 55.2 nN sum of vector magnitudes. This balance of forces was also determined over a population of images of cells ($n = 4$). It was found that the sum of the forces was 4.03 ± 2.59 nN. We also tested if the moments of the traction vectors were balanced. We calculated the moments of the x - and y -components, M_{yx} and M_{xy} , respectively, of the traction vectors relative to the origin. Ideally, balanced moments M_{xy} and M_{yx} should be equivalent and have a ratio of 1. The average ratio M_{yx}/M_{xy} of this population was 1.01 ± 0.10 ($n = 4$).

4. Discussion

We developed an indirect patterning approach that can be used to pattern features as small as 1 μm onto the surface of soft biomaterials. This approach can be used to pattern any soft

biomaterial as long as a bifunctional cross-linker can be incorporated into the gel that binds to amine groups that are prevalent on the surface of biologically relevant proteins. Thus, this technique adds topographical control of the placement of proteins on the surface of soft biomaterials to the already vast toolbox available for controlling their biochemical and mechanical properties. To demonstrate the efficacy of this approach, we showed that the fidelity of pattern transfer is sufficient to use the patterned dots for the measurement of CTFs, with average transfer errors of only 0.1 μm .

Our approach of indirectly patterning PAA gels provides important improvements over currently available approaches to measure CTFs. Until now, computational methods to convert substrate displacement into traction force either considered cell traction as a field [12,13,21], which is not consistent with cell force application through punctate focal adhesions, or utilized fluorescently tagged proteins that localize to focal adhesions [15,27] so that traction could be modeled as point forces [15,21]. A major advantage of our approach is that cell adhesion is inherently limited to patterned dots due to the non-fouling properties of PAA gel, so the traction forces can be modeled as discrete forces applied to individual dots. Importantly, our approach does not require cells to be removed from the PAA gel substrate in order to determine the reference image since it is inferred from the regularized dot pattern. This makes it possible to measure displacements and tractions in real time, and multiple fields of view can be imaged in high throughput. Compared with mPADs [17,31] and textured PDMS substrates [15], it is not currently possible to pattern individual posts or textured PDMS surfaces with 1 μm resolution. An additional and important advantage of our technique is the ability to pattern different biochemical cues with subcellular resolution. For example, recently described multicomponent surfaces that segregate integrin subtypes [32] could be transferred to PAA gels for measurement of CTFs on mixed substrates. Furthermore, our technique would be simple to adapt for strain application (as in Ref. [20]). Finally, our approach is computationally simple, and the code has been provided online for general distribution.

Accuracy, sensitivity and precision may also be important considerations for traction measurements. Microposts were originally incapable of resolving forces less than 12 nN [17]. However, this limit has been lowered significantly, and false positive displacements of <1 μm may be reduced [33]. Although we are unaware of any report that measures accuracy and precision using an ensemble of data acquired from posts in the absence of force, as we have done in Fig. 3 of our study, it appears that our limit of $\sim 0.3 \mu\text{m}$ is similar to microposts [33]. Similar limits have been reported for techniques involving PAA; however, the background varies with the magnitude of the traction due to the continuous field model and gel stiffness. An estimation of this limit for a gel with similar conditions used here would lead to a stress of approximately 110 Pa, which would translate into a force of 0.12 nN on a single dot [21]. It was recently shown that individual stress fibers in living cells have tensile forces of approximately 1.6 nN [34], which is higher than the force threshold used in our experiment, 0.98 nN. Thus, our technique is sufficient to image forces that are biologically relevant, since focal adhesions should have at least a single stress fiber attached.

Although we are unaware of published data on the success rate of creating microtextured PDMS, mPADs or micropatterning of PAA gels, the initial estimate of our success rate is approximately 50%, based on a number of trials done with transferring the pattern from glass coverslips to the PAA gels. Another important consideration with our approach relative to other techniques is that traction measurements depend upon imaging the fluorescently labeled pattern on the gel. The signal-to-noise ratio of the patterned spots compared to non-spot regions depends on a number of variable factors. Specifically, the camera settings, the amount of transferred protein and the labeling ratio of the printed protein (more fluorophores per molecule would decrease signal-to-noise ratio) cause

variations in signal-to-noise ratio. In well-patterned areas, the background is minimal and the fibronectin dots can be easily distinguished from the background. If the silicon master is improperly fabricated or damaged, or the PDMS stamp is damaged, both of which are generally characterized by large areas of printing errors, then it will be readily evident and the master and stamp can be discarded.

The indirect patterning approach does present some limitations. For example, our current computational approach assumes that force application does not result in the displacement of adjacent dots. Although this assumption is valid, given our dot spacing and the substrate stiffness used here, other applications may require closer dot spacing. Despite the lower spatial resolution of 5 μm obtained from this series of experiments, it is possible to achieve a greater resolution using a smaller inter-post spacing for the PDMS stamps. In comparison, mPADs have been created with 2 μm spatial resolution [33], while there have been claims that using microbeads embedded in a PAA substrate can achieve a spatial resolution of approximately 1 μm [21]. However, we anticipate improvement of the computational algorithm to account for adjacent dot displacement [21]. Secondly, this technique cannot, at its current stage, be used to detect forces being transmitted in the z -direction, perpendicular to the surface of the PAA gel. It has been reported that in PAA assays cells exert traction forces in three dimensions, even on relatively flat, two-dimensional substrates [35,36].

Our technique addresses the ambitious goal of making the measurement of CTFs simple, automated and real time. This approach is highly advantageous for a number of increasingly complex schemes. For example, we are currently utilizing the approach to make contrasting patterns of fibronectin and E-cadherin so that CTFs can be measured on biochemically distinct dots. Secondly, we are also working to culture cells in a sandwich culture, where one PAA surface has patterned dots in one fluorescent color while the opposing PAA surface is patterned with a second fluorophore. Thus, CTFs can be independently measured for both surfaces in this quasi-three-dimensional environment. Finally, we are adapting the cell mapping rheometry technique [20] to permit the application of defined one- and two-dimensional strains to cells. The advantage of using patterned PAA gels for cell mapping rheometry is that cell removal is not required, hence multiple cells in different fields of view can be imaged in real time during multiple cycles of strain and relaxation. Finally, we have made this approach generally available to any group with access to MATLAB, a fluorescent microscope and the ability to microcontact print glass surfaces.

5. Conclusions

The ability to independently tune the biochemical, mechanical and spatial/topographical properties of biomaterials provides the opportunity to control the cell fate for tissue engineering and regenerative medicine applications or to study cell behavior and improve our understanding of cell fate decisions. Patterning of PAA gels was shown here to improve measurement of CTFs, but this technique may be generally useful for patterning a variety of different biomaterials. We hope the simplicity of this technique and the availability of the tracking program makes measurement of CTFs widely available to the biomaterials community.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–4, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi: 10.1016/j.actbio.2011.08.013.

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Fig. 1.

A mold is created in PDMS that can stamp the desired pattern onto glass. Fluorescently labeled fibronectin in PBS is adsorbed onto the plasma-treated, hydrophilic stamp (A). After rinsing the surface, adsorbed fibronectin is then stamped onto the plasma-treated coverslip (B), thus leaving only the fibronectin from the raised features on the glass. This is then placed onto a PAA gel pre-polymer solution to create a gel “sandwich” between a second coverslip that is treated to form a covalent bond with the PAA polymer (C). After removal of the top coverslip, the pattern is covalently bond to the gel through NHS-ester and remains on the upper surface of the PAA gel (D), thus permitting cell attachment and gel contraction due to cell contractile forces (E). Fluorescent microscopic images are shown using a $\times 40$ water objective of a microcontact-printed pattern on glass (F) and a pattern after transfer onto the PAA gel (G). Scale bars = 30 μm .

Fig. 2.

A small drop of fluorescently labeled fibronectin was used to adsorb a circular region of fibronectin on a glass coverslip that was then transferred to the surface of a PAA gel containing NHS-ester during the polymerization phase. Fibroblast cells were then seeded onto the PAA gel and rinsed after 1 h to remove non-adherent cells. After 24 h of cell culture, both fluorescence (A) and bright-field images (B) of the interface of the fibronectin-coated circle and the non-passivated region of the PAA were acquired. The line in (B) represents the edge of the fibronectin island in order to determine cell features that extend beyond the pattern.

Fig. 3.

A patterned PAA gel was imaged under water and processed using the custom MATLAB code to generate apparent displacement vectors for each of the patterned dots. Displacement vectors are shown scaled by a factor of 10 times their actual length, corresponding to the color-coded displacement scale in μm (A). A cumulative frequency plot of all 450 dots that were analyzed in this field of view is shown (B), where the 25th, 50th and 75th percentile dots reside at 0.05, 0.94 and 0.12 μm , respectively. A histogram of the data was inset to demonstrate the distribution of the displacement vectors.

Fig. 4.

A fluorescence image of the fluorescently labeled fibronectin pattern (A) is overlaid with the bright-field image of a fibroblast cell after 24 h in culture on the patterned PAA gel (B). First, the analysis program identifies the locations of the dots in the fluorescence image and utilizes their predicted relaxed positions to calculate the displacement vector (colored by magnitude) for each dot (C). A cumulative distribution of all displacement vectors in the field of view is shown for both the area with the cell (blue) and a micropatterned area without cells (red; from Fig. 3C) (D). After thresholding, all dots with displacement vectors with magnitudes $\geq 0.3225 \mu\text{m}$ (3 pixels in length) are assigned values of 0; the remaining above-threshold displacement vectors are converted to traction vectors using Eq. (1) and are shown without the cell (E) or in the fluorescence/bright-field overlay (F). Scale bar for all images $15 \mu\text{m}$. The traction vectors are colored according to magnitude (E and F).