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Three-dimensional traction force microscopy for studying cellular interactions with biomaterials

J. Notbohm[a], J.-H. Kim[b], C. Franck[c], S. Maskarinec[d], D. Tirrell[d], A. Asthagiri[e], G. Ravichandran[a]*

[a]*California Institute of Technology, MC 105-50, Pasadena, CA 91125, USA*
[b]*Gwangju Institute of Science and Technology, 261 Cheomdan-gwagiro, Buk-gu, Gwangju 500-712 Republic of Korea*
[c]*Brown University, Engineering Box D, Providence, RI 02912, USA*
[d]*California Institute of Technology, MC 210-41, Pasadena, CA 91125, USA*
[e]*Northeastern University, 360 Huntington Ave, Boston, MA 02115, USA*

Abstract

The interactions between biochemical and mechanical signals during cell adhesion, migration, spreading and other processes influence cellular behavior. Three-dimensional measurement techniques are needed to investigate the effect of mechanical properties of the substrate on cellular behavior. This paper discusses a three-dimensional full-field measurement technique that has been developed for measuring large deformations in soft materials. The technique utilizes a digital volume correlation (DVC) algorithm to track motions of sub-volumes within 3-D images obtained using laser scanning confocal microscopy. The technique is well-suited for investigating 3-D mechanical interactions between cells and the extracellular matrix and for obtaining local constitutive properties of soft biomaterials. Results from the migration of single fibroblast cells on polyacrylamide gels and their implications for cell motility models are discussed. The implications that the traction distributions of epithelial cell clusters have on the inhibition of proliferation due to cell contact and scattering of cells in a cluster are discussed. These results provide insights on force fields generated by cells and the role of the mechanical properties of the substrate on cellular interactions and mechanotransduction. Analytical solutions and finite element simulations are used to elucidate the mechanics of cellular forces exerted on the extracellular matrix.

Keywords: Traction force microscopy; confocal microscopy; digital volume correlation

* Corresponding author. Tel.: +1-626-395-4525; fax: +1-626-449-2677.
E-mail address: ravi@caltech.edu

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1. Introduction

Mechanical tractions applied by cells have been shown to affect cell growth [1], differentiation [2], and migration [3]. As a result of these studies, the measurement of the forces that cells apply has been a topic of significant interest. Most previous measurements of the tractions applied by cells have focused on single cells in two dimensions (2D) by using surface wrinkling [4], particle tracking traction force microscopy (TFM) [5], and flexible micropillar arrays [6]. While these methods have led to insights into the relationship between cellular tractions and biochemical cellular processes, the restriction of single cells in 2D has left unanswered questions pertaining to the three dimensional (3-D) tractions that cells apply and the way in which multiple cells interact mechanically.

We have developed a new technique for three-dimensional traction force microscopy (3DTFM) by combining confocal microscopy with digital volume correlation algorithm [7]. This technique allows us to quantify cellular traction forces in all three dimensions (3D) [8,9]. This paper discusses the use of 3DTFM to quantify tractions in two cellular systems: (1) 3T3 fibroblasts and fibronectin-functionalized polyacrylamide gels and (2) clusters of MDCK epithelial cells on substrates of different stiffness.

2. Methods

2.1. Polyacrylamide films

Three different polyacrylamide gels were used as the substrate in the TFM experiments. The gels were made with 40% stock solution polyacrylamide (Bio-Rad, Hercules, CA) mixed with deionized water to a concentration of 10% polyacrylamide. Three different concentrations of bisacrylamide, 0.0075%, 0.04%, and 0.10% were made from a 2% stock solution (Bio-Rad). The Young's moduli of the gels were determined by using a custom-built compression experiment described previously [7] to be 0.82±0.23 kPa, 7.1±0.4 kPa, and 17.4±1.50 kPa, respectively. 1 μm fluorescent microbeads (Invitrogen, Carlsbad, CA) were suspended in the gels at a concentration of approximately 0.2%, and the gels were pipetted onto a 25 mm glass coverslip. A 15 mm glass coverslip was placed onto the gel before it polymerizes, creating a polyacrylamide film with a thickness of approximately 75 μm.

The 15 mm coverglass was then removed, and the polyacrylamide gels were functionalized with fibronectin (Sigma-Aldrich, St. Louis, MO) using the heterobifunctional cross-linker sulfo-SANPAH (Pierce, Rockford, IL) as described previously [10].

2.2. Cell culture

Swiss 3T3 fibroblasts transfected with green fluorescent protein were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/mL streptomycin, and 50 μg/mL penicillin. For all experiments, cells were first treated with Mitotracker Deep Red (Invitrogen) for 45 minutes before passaging with trypsin. Cells were plated at a concentration of approximately 40,000 cells/coverslip and were incubated on samples for 8-12 hours before imaging.

Madin Darby canine kidney cells were grown in DMEM with HEPES and L-glutamine (Invitrogen) and 10% (v/v) fetal bovine serum (Invitrogen). Prior to experiments, cells were placed in serum-starved medium for 24 hours and then stained with Mitotracker Deep Red (Invitrogen) for imaging.

2.3. Confocal microscopy

A Nikon C1 confocal microscope on a TE2000 stand was used with either a 40x 0.6 NA air objective (for imaging the 3T3 fibroblasts) or a 60x 1.45 NA oil objective (for imaging the MDCK cells). A 488 nm argon laser and a 633 nm helium-neon laser were used to excite fluorescence. 512×512 pixel images were collected in a field of view ranging from 150×150 μm^2 to 200×200 μm^2. Confocal stacks were collected with a step size of 0.4 μm. A diagram of the experimental setup is shown in fig. 1. All confocal imaging was performed at 37°C using a custom-build enclosure heated with an Air Therm ATX heater (World Precision Instruments, Sarasota, FL).

2.4. Deconvolution

While the confocal microscope provided better in-plane (x-y) and axial (z) resolution than a widefield microscope, the resolution in the axial direction was still lower than in the in-plane direction. As a result, the spherical fluorescent particles were imaged as ellipsoids that were elongated in the axial direction. To improve the axial resolution, the Lucy-Richardson deconvolution algorithm was used to deconvolve the image from the axial point spread function of the confocal microscope as described previously [7].

2.5. Digital volume correlation

To calculate the displacements, strains, and stresses applied by the cells to the polyacrylamide substrate, a digital volume correlation (DVC) algorithm [7] was used to calculate the full 3D displacement profile within the substrate. Briefly, this algorithm picked a 64×64×64 voxel subset of the image. In the reference configuration, the intensities associated with this subset were called $f(\boldsymbol{x})$. It was assumed between the reference and deformed images that the subset underwent a rigid body translation with mapping $\boldsymbol{y}=\boldsymbol{x}+\boldsymbol{c}$. By using this mapping along with the intensity distribution of the deformed image $g(\boldsymbol{y})$, the DVC algorithm solved the equation $f(\boldsymbol{x})=g(\boldsymbol{y})=g(\boldsymbol{x}+\boldsymbol{c})$ using a cross-correlation in the Fourier domain for $\boldsymbol{c}$, which corresponded to the 3D displacement vector of the subset. The DVC algorithm then moved on to another subset and repeated this process until the displacements throughout the substrate were calculated. Noise floor experiments were performed wherein a polyacrylamide gel with beads but no cells was imaged. Translations and strains were computationally applied to the speckle pattern formed by the beads. It was found that the displacement noise floor was approximately 0.1 μm. The strain noise floor was found to be in the range of 0.5% to 1% strain for the out-of-plane (z) components of strain, which are the strain components discussed here.

2.6. Finite element model

A simple finite element model of the MDCK cell cluster traction experiments was created in ABAQUS 6.9 (Simulia, Providence, RI). The model is similar to previously-published models on contraction of a cluster of cells [11]. In the model, the substrate was modeled with linear, elastic 8-node brick elements with a Young's modulus of either 7.1 or 17.4 kPa and Poisson's ratio of 0.48. The MDCK cells were also modeled as linear, elastic 8-node bricks. Each cell was made up of 25 brick elements with a Young's modulus 1 kPa and a Poisson's ratio 0.48 sitting on the top of the substrate. The cell elements were connected to the substrate and to each other with linear springs of varying spring stiffness. In-plane contraction of the cells was simulated using a contractile thermal strain in the cell elements.

3. Results and discussion

3.1. Migrating fibroblasts

Digital volume correlation and confocal microscopy have been combined to quantify the time resolved x-, y- and z-components of displacements exerted by 3T3 fibroblasts as they move atop a soft polyacrylamide substrate (E~1 kPa) coated with the adhesion ligand protein fibronectin. Because the full 3D displacement profile of the substrate is known, the tractions can be computed directly using this method. To compute tractions, the displacement field is differentiated by fitting a 3x3x3 window of points to a trilinear equation to calculate the strain tensor. Since the polyacrylamide substrate is linear, elastic, and nearly incompressible, the incompressible form of Hooke's Law is used to compute the stress tensor from the strain tensor. The Cauchy relation is then used to compute the tractions applied by the cells to the top surface of the polyacrylamide gel.

Fig. 1(b) shows a cross-sectional view of a typical confocal stack with simultaneous visualization of the fluorescent markers (red) and GFP vector encoded F actin (green) in a 3T3 fibroblast cell. As the cell explores the substrate and probes its resistance to deformation, displacements are determined by comparing successive stacks. Importantly, the DVC method can map such displacements in all three dimensions by comparing uniquely defined cubic subsets within confocal stacks that are obtained sequentially [7]. This technique can detect both translational and rotational changes and is valid for large deformations.

Detection of both normal and in-plane displacements—as opposed to consideration of only in-plane (2D) forces—is necessary to fully analyze cellular forces in 3D. Fig. 2 shows the extent of cell-induced deformation as a function of depth within the substrate, and it shows clearly that the z-axis displacement can be comparable to, or greater than, the in-plane displacements. In the images in fig. 2(a), a slice along the long axis of the cell is shown, and the cell has been rendered in 3D and superimposed to correlate its position with the observed displacements.

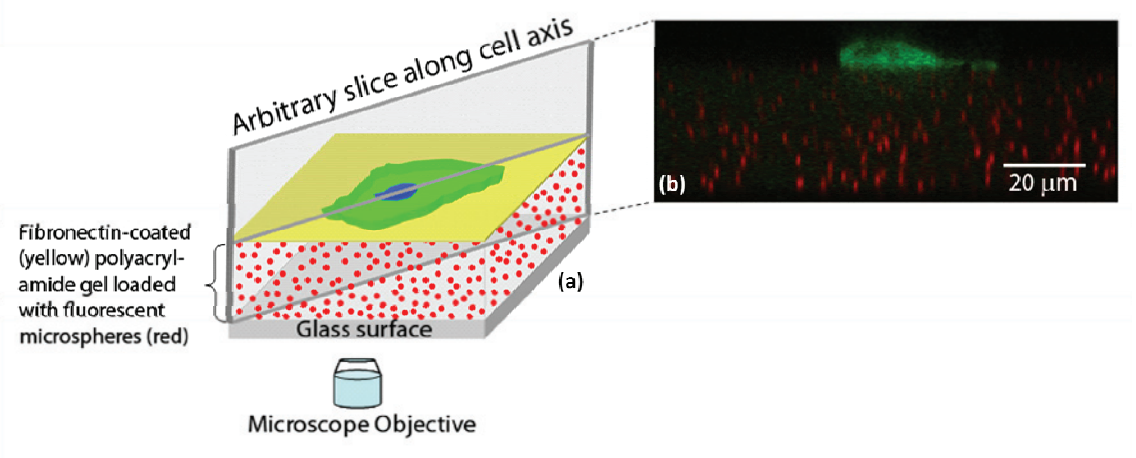

Figure 1: A sketch of the experimental setup including the microscope objective, the functionalized polyacrylamide gel, and the cell is shown in (a). An image acquired using the confocal microscope of the cell sitting on the polyacrylamide substrate is shown in (b) [8].

The colored displacement contours show that larger displacements are concentrated near the top surface of the gel, and that these displacements decay from the top surface through the depth. The decay

profiles of individual displacement components as well as the total displacements for a substrate of 40 μm thickness are displayed as line plots in fig. 2(b). The plot is generated by selecting one position along the thickness cross-section of the sample near the leading edge of the migrating cell.The total displacement profile at a given time point and slice location can be dominated by either in-plane or normal displacements, demonstrating how force-mediated deformations fluctuate during cell movement. It is worth noting that deformation of the substrate can be detected at substantial depths below the top surface of each sample, and that this effect can be especially important for thin gels. The surface traction forces ($pN/\mu m^2$), which are computed directly from the displacements, are shown in fig. 2(c). The data is collected over a period of time (up to 24 hours, not shown here) and suggests the cells dynamically change the tractions they apply during migration.

3.2. Epithelial cell clusters

In a cultured cell cluster, cells adhere both to each other and to the substrate beneath them. While TFM experiments can only measure the tractions applied by the cells to the substrate, these experiments can still prove useful for characterizing the way in which cells interact with each other mechanically. Displacements applied by MDCK cells are measured using confocal microscopy and DVC. Since it turns out that the cells apply tractions of similar magnitude to this method's noise floor in the axial direction (~100 Pa), only the in-plane data is considered in this study. To compute tractions, the in-plane displacements at the top of the gel are used as a boundary condition in a finite element computation. This finite element computation circumvents the need for an inverse-Boussinesq formulation which requires data smoothing [12] and additional analysis to account for the finite thickness of the substrate [13]. The gel is modeled as a linear elastic material with a Young's modulus of either 7.1 kPa or 17.4 kPa. After putting in the displacement boundary condition at the top of the gel, the equilibrium problem is solved using the finite element software Abaqus 6.9 (Simulia, Providence, RI). The in-plane tractions applied by the cells to the polyacrylamide substrate are then computed using the Cauchy relationship.

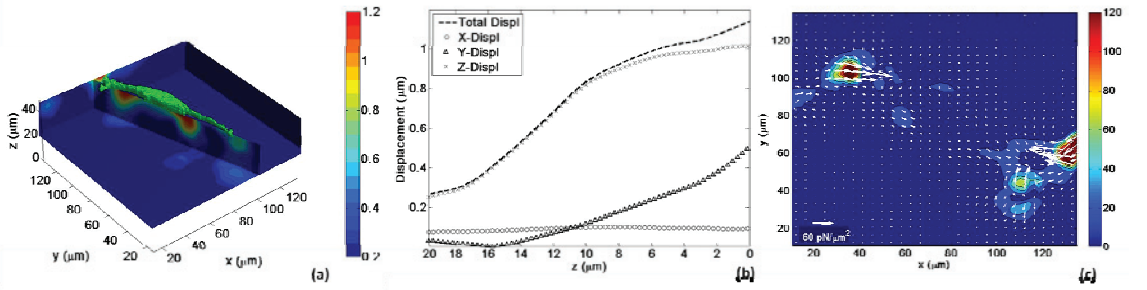

Figure 2: A migrating fibroblast applies displacements to the substrate beneath it (a). The displacements under the cell at the top of the substrate (b) indicate that the cell applies displacements in all directions. The tractions applied by the cell are shown in (c). The colours indicate the 3D traction magnitude, and the arrows show the direction of the in-plane (*x*-*y*) traction components. The data show the cell pulls inward (indicated by white arrows) with a maximum magnitude of 120 $pN/\mu m^2$ [8].

Representative in-plane traction contour plots are shown in fig. 3 for a soft substrate (fig. 3a) and a stiff substrate (fig. 3b). The data show that the cell cluster pulls inward on the polyacrylamide substrate. As can be seen in the figure, it appears that the cell cluster on the stiffer substrate applies a smaller traction magnitude than the cell cluster on the softer substrate. However, repeated experiments have shown no statistically significant difference between the maximum traction magnitudes applied by cells on either the stiff or soft substrates.

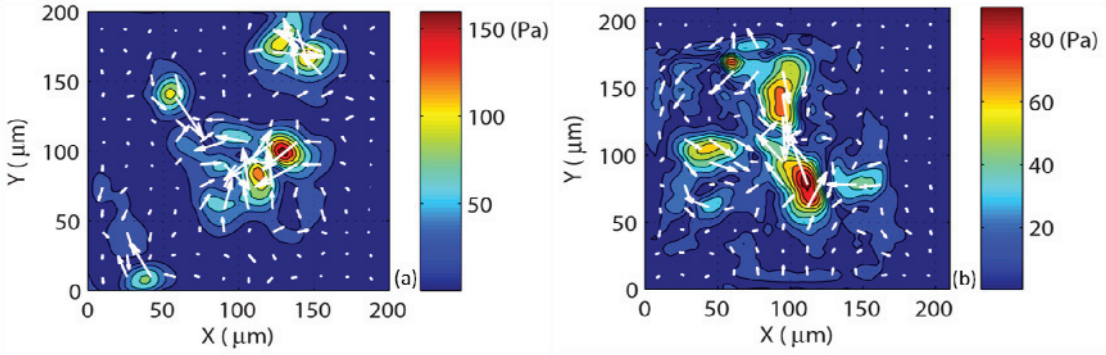

Figure 3: In-plane tractions applied by MDCK cell clusters to the soft (a) and stiff (b) polyacrylamide substrate are shown by the colour contours. The arrows indicate the direction of traction.

To better understand this observation, a finite element (FE) model is developed. In the FE model, shown in fig. 4a, cell-cell and cell-substrate adhesions are represented by linear springs with adjustable stiffness. The tractions applied by the cell clusters are simulated by an isotropic thermal strain applied to the cell elements. The level of thermal contraction and the values of cell-cell and cell-substrate spring stiffness are calibrated so as to match the traction magnitudes of the TFM experiments on the soft gel, and simulated tractions are plotted in fig. 4b. To model the TFM experiments on the stiff gel, the Young's modulus is changed to match the experimentally-measured polyacrylamide substrate Young's modulus, and the FE model is solved again. Tractions for the stiff gel are shown in fig. 4c. As can be seen by comparing figs. 4b and 4c, there is little difference between the traction magnitudes, which agrees with our experimental results.

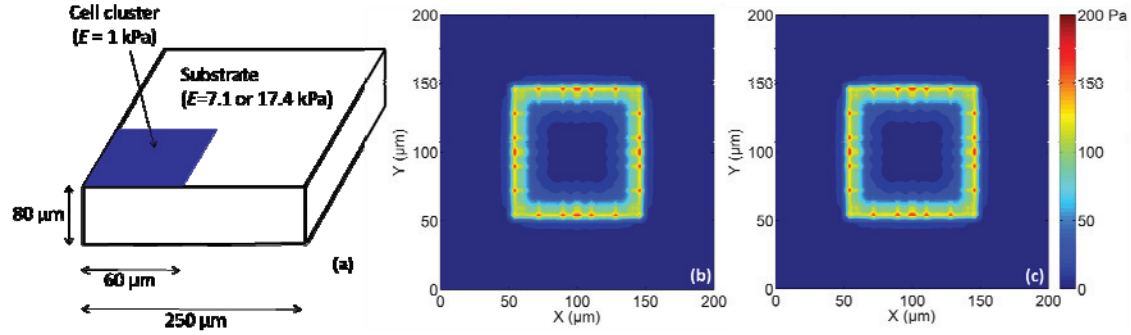

Figure 4: (a) A schematic of the FE model shows the dimensions used. The cells within the cluster are attached to their neighbours and to the susbtrate with linear springs. Thermal contraction of the cell elements is used to simulate the cell contraction. The spring stiffnesses and level of thermal contraction are calibrated to match the experimenal traction data on a soft gel (E = 7.1 kPa) shown in fig. 3b, and the tractions are plotted in (b). The FE model is re-solved using a value of Young's modulus that matches the stiff gel (E = 17.4 kPa), and the tractions are plotted in (c). In both (b) and (c), the tractions are directed inward, toward the centre of the image.

The FE model also predicts that a well-adhered cluster of cells applies the largest traction at the exterior of the cluster. This implies that to maintain a force balance, the interior cells must apply tractions to each other rather than the substrate beneath them. This observation could have interesting biological implications and could be a topic of further study.

4. Conclusions

While the technique of traction force microscopy has been used extensively in the past decade, there has been a lack of extending this technique to 3D and more complicated systems, such as clusters of cells. Here, we have presented a 3D TFM technique and have showed how it can be applied to migrating fibroblasts and epithelial cell clusters. The results from the experiments with migrating fibroblasts show that cells on compliant substrates apply force in all three dimensions, which highlights the importance of 3D techniques. The experiments with the epithelial cell clusters combined with the finite element model have demonstrated the importance of the stiffness of the extracellular environment on cellular behaviors such as cell-cell and cell-substrate adhesion.

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