

Development of Micropost Force Sensor Array with Culture Experiments for Determination of Cell Traction Forces

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Cell traction forces (CTFs) are critical for cell motility and cell shape maintenance. As such, they play a fundamental role in many biological processes such as angiogenesis, embryogenesis, inflammation, and wound healing. To determine CTFs at the sub-cellular level with high sensitivity, we have developed high density micropost force sensor array (MFSA), which consists of an array of vertically standing poly(dimethylsiloxane) (PDMS) microposts, 2 μm in diameter and 6 μm in height, with a center-to-center distance of 4 μm . In combination with new image analysis algorithms, the MFSA can achieve a spatial resolution of 40 nm and a force sensitivity of 0.5 nN. Culture experiments with various types of cells showed that this MFSA technology can effectively determine CTFs of cells with different sizes and traction force magnitudes. *Cell Motil. Cytoskeleton* 64: 509–518, 2007. © 2007 Wiley-Liss, Inc.

Key words: cell traction force; micropost array; finite element method; image analysis; smooth muscle cells; fibroblasts

INTRODUCTION

Adherent cells exert contractile forces on substrate or extracellular matrix (ECM) [Janmey and Weitz, 2004]. These forces are generated by the actomyosin contractile machinery of a cell and centripetally applied to the substrate through focal adhesions (FAs) [Burton et al., 1999]. Since these forces permit cells to migrate, they are referred to as cell traction forces (CTFs) [Riveline et al., 2001; Bershadsky et al., 2003; Huang et al., 2004]. CTFs enable cell motility and maintenance of cell shape, therefore playing an essential role in many biological processes such as angiogenesis, embryogenesis, inflammation, and wound healing.

A variety of techniques have been developed to determine CTFs at the sub-cellular level; the majority of

them use various types of deformable substrates. Continuum substrates, including thin silicone polymer membranes [Harris et al., 1980; Oliver et al., 1995; Balaban et al., 2001] and fluorescent beads-embedded polyacryl-

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amide gels [Dembo and Wang, 1999; Beningo et al., 2002; Wang et al., 2002], have been commonly used for measuring CTFs. On these substrates, however, the strain propagates across the surface; therefore, the displacement of any point on the substrate is coupled with the displacements of its neighbors. In addition, the location of CTFs is uncertain, and magnitude of forces may be affected by the algorithm used in computation of CTFs.

These limitations of using continuum substrates to determine CTFs can be overcome by the use of discrete substrates, such as micropost arrays [Tan et al., 2003; du Roure et al., 2005; Lemmon et al., 2005; Saez et al., 2005; Zhao and Zhang, 2005] and microfabricated cantilevers [Galbraith and Sheetz, 1997; Park et al., 2005; Yang and Saif, 2005]. On these substrates, each force sensing unit independently detects the CTF at the site where it contacts the cell. The micropost array-based technique appears to be among the most effective of these approaches for measuring CTF at the sub-cellular level. Specifically, when a cell resides on an array of microposts, it bends them at the top. Once the lateral deflections of the microposts are obtained by image acquisition and analysis, CTFs can be determined based on the beam theory [Timoshenko and Woinowsky-Kreiger, 1959]. Besides CTF measurement, micropost arrays have also been used for assembling freely suspended two-dimensional actin cortex network [Roos et al., 2003] and myofibrils [Zhao and Zhang, 2006], electrophoresis separation of DNA [Inatomi et al., 2003], and studying dynamics of molecular motor-driven microtubule gliding and microtubule network formation [Roos et al., 2005].

However, current applications of micropost array technologies are mainly limited to cells that generate large contractile forces, such as cardiac fibroblasts or myocytes [Balaban et al., 2001; Zhao and Zhang, 2005] and smooth muscle cells [Tan et al., 2003], or cell monolayers which possess accumulative forces at the edge significantly greater than those of individual cells [du Roure et al., 2005]. To extend the application of this technology to cells of smaller size or producing weaker contractile forces than smooth muscle cells, such as tendon fibroblasts, it is necessary to develop micropost arrays with higher density and smaller compliance. In addition, the classical beam theory that the majority of existing studies use to determine the deflection of a micropost is only valid for small deflections. We have developed high density micropost force sensor arrays (MFSAs) for measuring CTFs with high sensitivity and accuracy even under the condition of large micropost deflections. Herein, we report the fabrication and characterization of MFSAs and development of new image analysis methods for determining micropost displacements with high resolution and hence high sensitivity in CTF detection. The application of this MFSA technology to determine

CTFs of various types of cells in our culture experiments is also presented.

MATERIALS AND METHODS

Fabrication of MFSAs

The MFSA was fabricated by replica molding technique using a silicon wafer as the mold to cast PDMS (Fig. 1). The wafer was fabricated using standard photolithography followed by a Bosch etching process (MEMS and Nanotechnology Exchange, Reston, VA). To facilitate the release of PDMS, the wafer surface was treated with a vapor of (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (Gelest, Morrisville, PA) for 12 h to render it high surface energy. After that, a 10:1 (w/w) mixture of PDMS prepolymer (Sylgard 184, Dow Corning, Midland, MI) was poured onto the wafer, cured at 65°C for 24 h, and then peeled off from it.

Phase Contrast Microscopy and Scanning Electron Microscopy

Phase contrast microscopy with Eclipse TS100 or Eclipse TE2000-U microscopes (Nikon, Japan) was used to image the array of microposts with or without cells, whereas SEM was used to reveal the morphology and geometry of individual microposts. Prior to SEM observation on a JSM6330F system (JEOL, Japan), the MFSA specimens were sputter-coated with a layer of gold (3.5 nm in thickness) to render them electronic conductivity.

Finite Element Method Analysis

FEM was used to determine the deflection-force relationship of the microposts. FEM facilitates the incorporation of the finite strain effects on the deflection-force relationship of the microposts. To avoid errors caused by neglecting the transverse shear deformation of Euler-Bernoulli's beam theory, the Timoshenko beam FE model was used. A direct result is that a plane normal to the beam axis before deformation does not have to remain so after it is deformed. The Young's modulus of PDMS is 2 MPa, and the Poisson's ratio is 0.49. The micropost cantilevers used in this study have the same height-to-diameter ratio of 3. The micropost was meshed with an element size of 0.5 μm . The bottom of the micropost was fixed, a point force was applied to the center of the top cross section (Fig. 2A). The deflections of the top corresponding to different levels of point forces were computed using ANSYS 10.0 software (ANSYS, Inc., Southpointe, PA), with the finite strain-capable Timoshenko beam element, Beam188. The obtained deflections vs. forces follow a quadratic relationship (Fig. 2B).

Image Acquisition and Analysis

First, a set of images of microposts were captured in a certain region of the MFSA using phase contrast microscopy. Using these images, the deflections of microposts were determined by image analysis approaches as further discussed below. Because of the waveguide effect of the microposts, the individual microposts appear as distinct white clots in the microscopy images. The deflection of micropost is therefore termed “displacement” in an image. By focusing at either the top or the bottom of microposts, two images were obtained and denoted as the “force-loaded” image and the “force-in-

dependent” image, respectively. A fluorescence image of a cell on the micropost array was also recorded through fluorescence microscopy to define the cell region. In some experiments, an image was taken after the cell was removed, in which the positions of microposts represent their initial positions. This image is therefore referred to as the “null-force” image. All images were recorded in 8-bit non-compressed TIFF format. As described below, two novel image acquisition and processing approaches were developed and integrated into a MATLAB program to determine the displacements/deflections of individual microposts.

The first approach, termed “Top-Bottom” method, determined the displacements by comparing the top positions of microposts with those at the bottom (Fig. 3). Three images, namely, a “force-loaded” image at the micropost top (Fig. 3A), a “force-independent” image at the micropost bottom (Fig. 3B), and a fluorescence cell image (Fig. 3C), were utilized for image analysis. Our algorithm determined the centroids of both top and bottom images of the microposts, which appeared as distinct white clots, and then computed the displacement as the difference between these two for each micropost (Fig. 3D). In order to minimize possible displacements of the centroids due to image shifting during image acquisition the following steps were taken. A mean displacement “drift vector” was determined by averaging the displacements of centroids outside the cell region. Next all the centroids in the “force-loaded” image were corrected by the drift vector. Finally, the displacement of each micropost was determined by subtracting the position of the centroid corresponding to that micropost in the “force-independent” image from its counterpart in the “force-loaded” image.

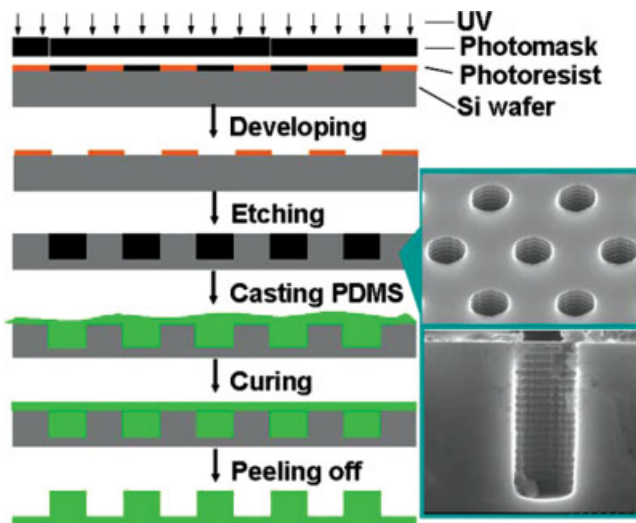


Fig. 1. Schematic of the photolithography and replica molding processes for MFSA fabrication.

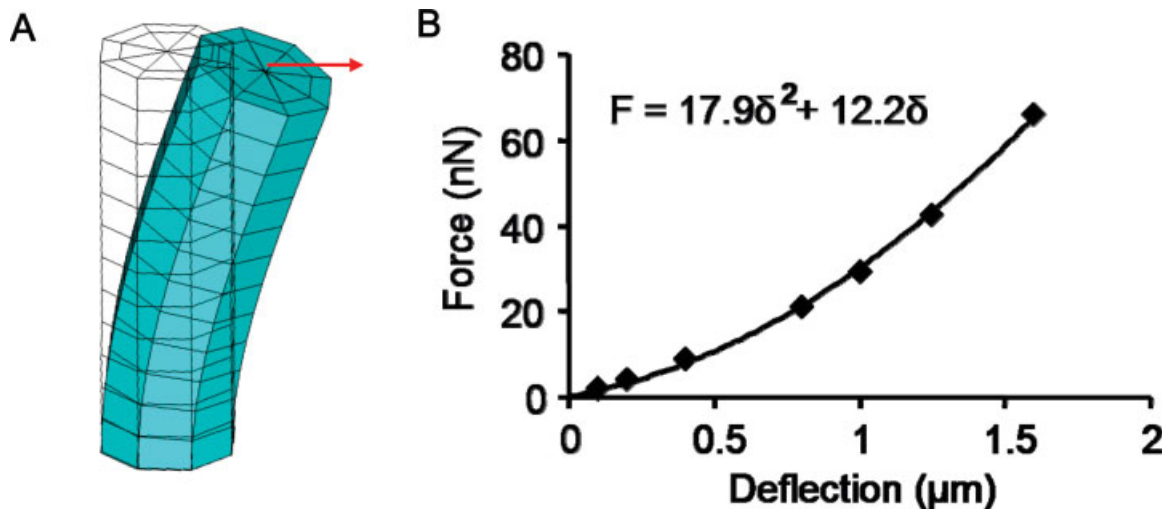


Fig. 2. (A) Schematic of FEM model for a micropost with a lateral force exerted on its top. (B) The relationship between the micropost deflection and the lateral force determined by FEM.

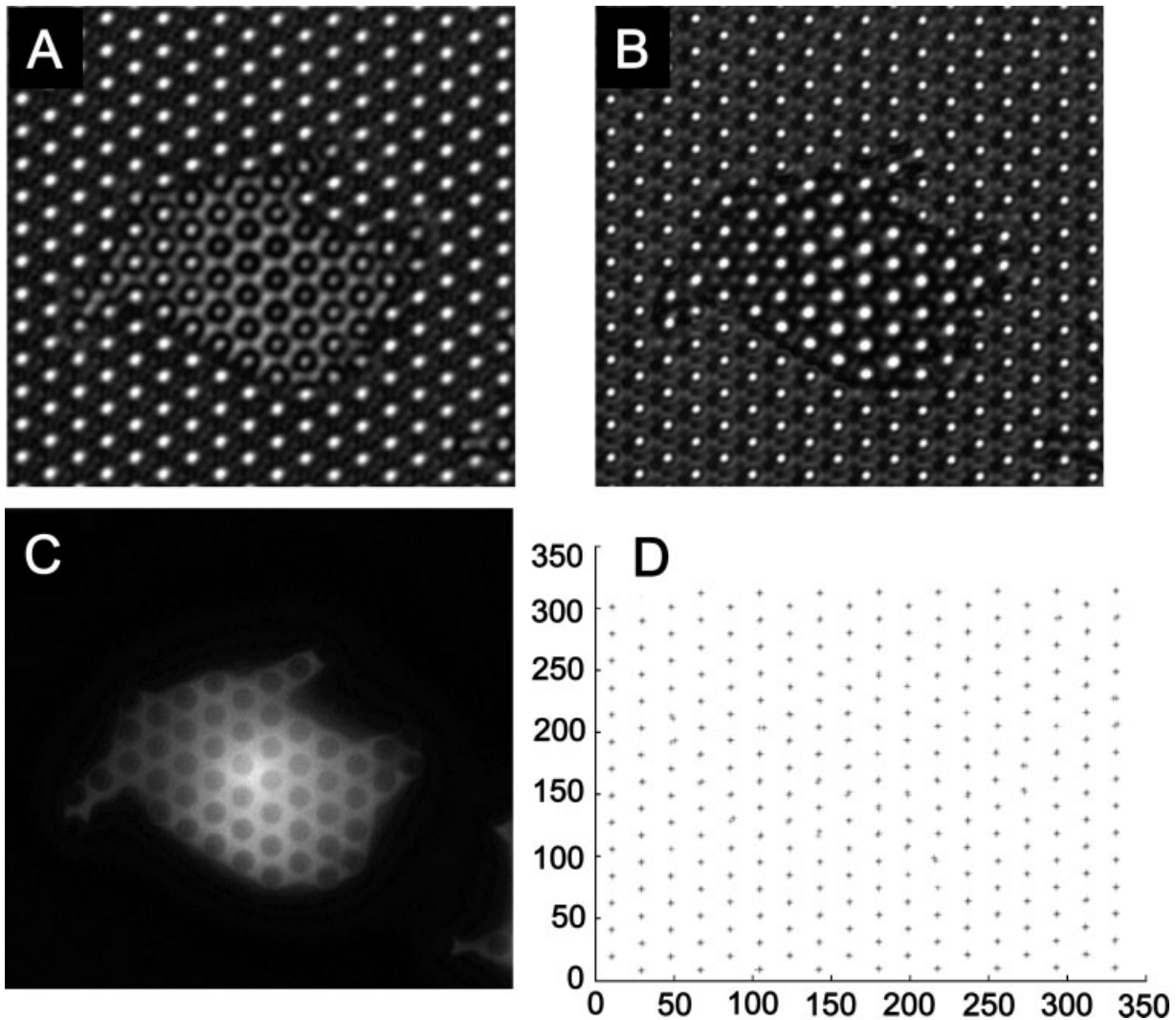


Fig. 3. “**Top-Bottom**” method for CTF measurement. (A) The “*force-loaded*” image of microposts. (B) The “*force-independent*” image of microposts. (C) The fluorescence image of the cell. (D) The centroids being overlaid and matched from the “*force-loaded*” image and the “*force-independent*” images, respectively.

In the other approach denoted as the “*Before-After*” method, the displacements of microposts were determined by comparing the positions of microposts in the “*force-loaded*” image (Fig. 4A) with those in the “*null-force*” image (Fig. 4B). The basis for this approach is that initial small micropost deflections exist; therefore, they should be excluded to accurately determine the displacements of the microposts due to the action of CTFs.

Culture Experiments with MFSAs

To show the effectiveness of MFSAs in measuring CTFs, we performed culture experiments with several types of cells. These included human patellar tendon fibroblasts (HPTFs) derived from human tendon samples (IRB No. 0108109, University of Pittsburgh), human

skin fibroblasts (HSFs) from ATCC (Manassas, VA), and smooth muscle cells (SMCs) from rat aorta (courtesy of Dr. W. R. Wagner, University of Pittsburgh). The MFSAs were first treated with air plasma for 4 min and subsequently coated with 50 $\mu\text{g}/\text{ml}$ human plasma fibronectin (Chemicon International, Temecula, CA) for 1 h before cells were seeded. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere. In some experiments, cells were visualized by incubation with 2 μM calcein-green AM ester (CGAM, Sigma-Aldrich, St. Louis, MO) for 5 min. Removal of cells from MFSAs was achieved by applying a 10% bleach solution for 1 min.

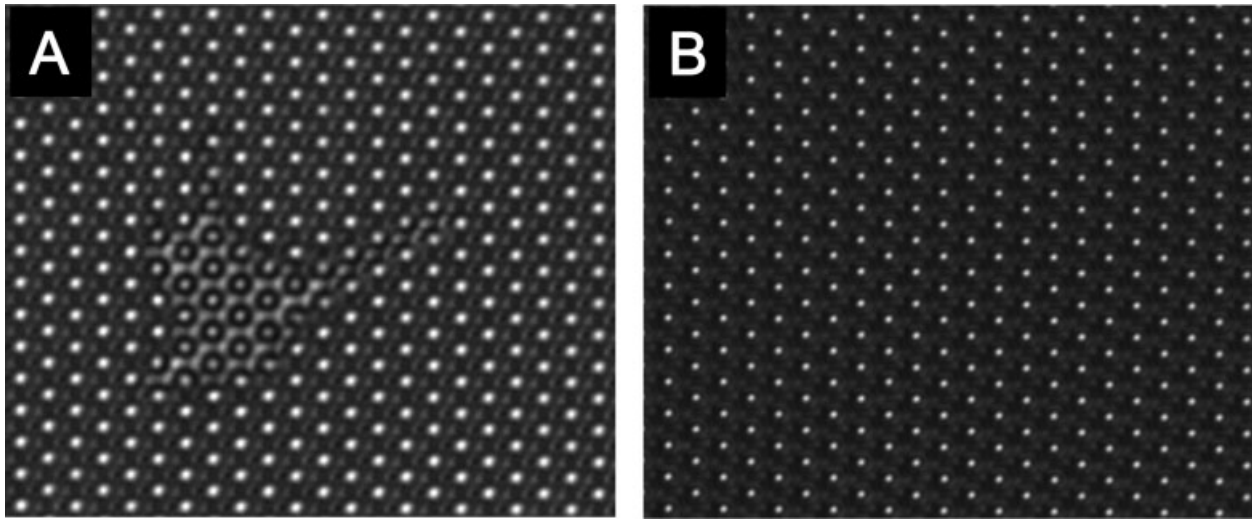


Fig. 4. “Before-After” method for CTF measurement. (A) The “force-loaded” image of microposts. (B) The “null-force” image of microposts.

Staining Actin Filaments and Focal Adhesions

A FAK100 kit (Chemicon International) was used to stain actin filaments and focal adhesions. Briefly, the cultured cells were fixed with phosphate-buffered 4% paraformaldehyde (FD NeuroTechnologies, Ellicott City, MD) for 10 min, followed by incubation in a Blotto buffer (4% dry milk and 0.4% Triton X-100 in PBS) for 1 h. The primary antibody (anti-vinculin) was diluted in Blotto (dilution 1:100) and applied for 1 h. Then the sample was washed three times for 10 min with Blotto. The secondary antibody (goat anti-mouse, FITC-conjugated, dilution 1:200) and TRITC-conjugated phalloidin (dilution 1:200) were applied simultaneously for 1 h at room temperature. The sample was then washed three times for 10 min with Blotto, followed by incubation with BisBenzimide H33258 (Sigma-Aldrich) solution for 2 min. Finally, the specimen was mounted, and fluorescence images were recorded on a fluorescence microscope (Eclipse TE2000-U, Nikon, Japan).

RESULTS

The Morphology and Geometry of MFSAs

High quality MFSAs were obtained in this study (Fig. 5A). The MFSAs consist of an array of vertically standing PDMS microposts, 2 μm in diameter and 6 μm in height, with a center-to-center distance of 4 μm . Because of the physical characteristics, the microposts behave as individual waveguides and thus result in high contrast images with phase contrast microscopy (Fig. 5B). The high contrast images afford accurate measurement of the displacements of the microposts.

The Micropost Deflection-Force Relationship

The relationship between micropost deflection and the laterally exerted point force at the top of micropost was obtained by FEM analysis. For a large deflection ($>0.5 \mu\text{m}$), the relationship between the deflection of micropost and force is non-linear (Fig. 2B). The deflection-force relationship was found to be well fitted by a quadratic function.

The Resolution of Deflection and Force Sensitivity of MFSAs

As preceding discussion elucidates that in the determination of the micropost deflection, the “Top-Bottom” method (Fig. 3) is subjected to a shortcoming by not considering the initial pre-existing deflection as the “Before-After” method (Fig. 4) does. This affects the level of resulting displacement resolution. In our study, we have achieved micropost displacement resolutions as high as 70 and 40 nm, respectively, for “Top-Bottom” and “Before-After” approaches. These further translated into force sensitivities at the level of 0.9 and 0.5 nN, respectively.

Cell Spreading and Proliferation on MFSAs

When cells were plated to MFSAs, they attached to and spread on the MFSAs’ top surface. Figure 6A shows that HSFs adhered to the MFSAs surface within 1 h and developed fully spread morphology within about 8 h. The cells on the MFSAs developed numerous stress fibers and focal adhesions that were formed exclusively at the contact sites between cell and the microposts (Fig. 6B). Note that, however, the immuno-stained images of cells

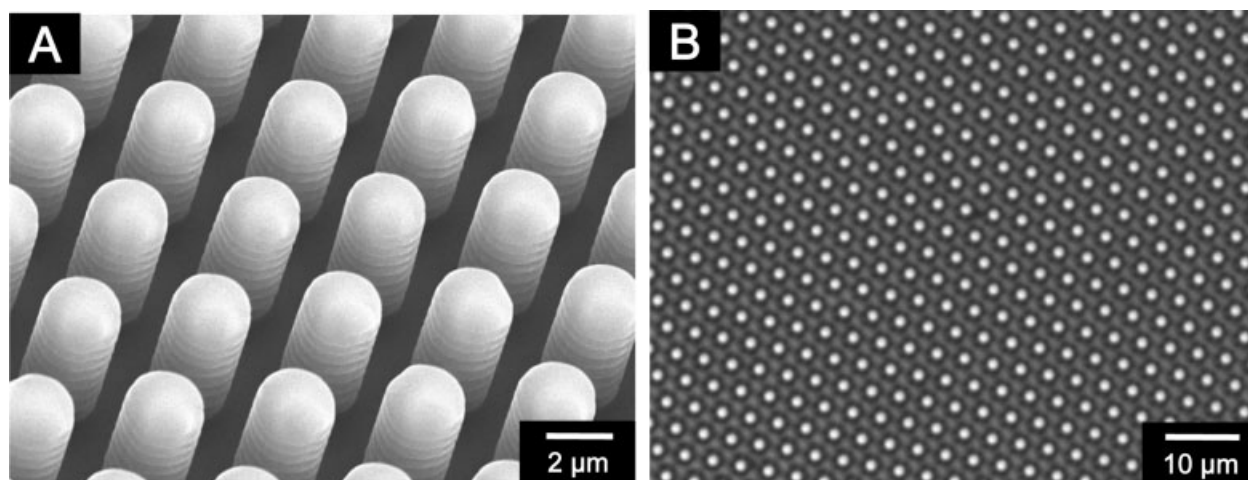


Fig. 5. (A) SEM image of MFSA. (B) phase contrast microscopy image of MFSA.

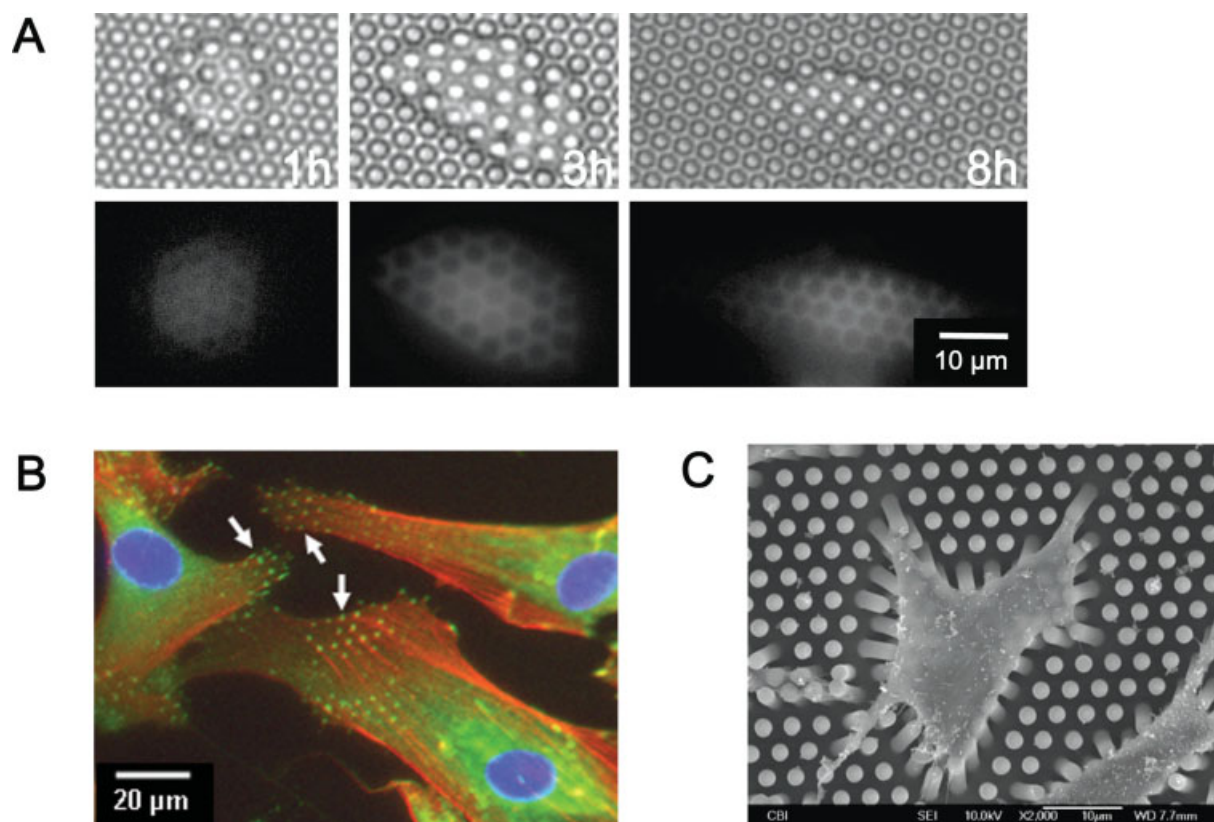


Fig. 6. (A) Time course of a HSF attaching to and spreading on MFSA. The top row shows the phase contrast images and the bottom row shows the fluorescence microscopy images of the same cells that were labeled using CGAM. (B) Fluorescence microscopy image of HSFs cultured on a MFSA. TRITC-conjugated phalloidin (red), anti-

vinculin monoclonal antibody and FITC-conjugated secondary antibody (green), and BisBenzimide H33258 (blue) were used to reveal actin filaments, focal contacts, and nuclei, respectively. Note that the green dots (indicated by white arrows) are focal contacts on the top of microposts. (C) SEM image of rat aorta SMCs adhering to the top of MFSA.

on the MFSA can serve only as a graphical description of focal adhesions and stress fiber bundles because the fixation process can dramatically change the internal forces in the cells.

Also, it was noted that for all types of cells used in this study, there was no apparent difference in morphology between cells on MFSA and those on smooth PDMS surfaces; however, the cells on MFSA appeared

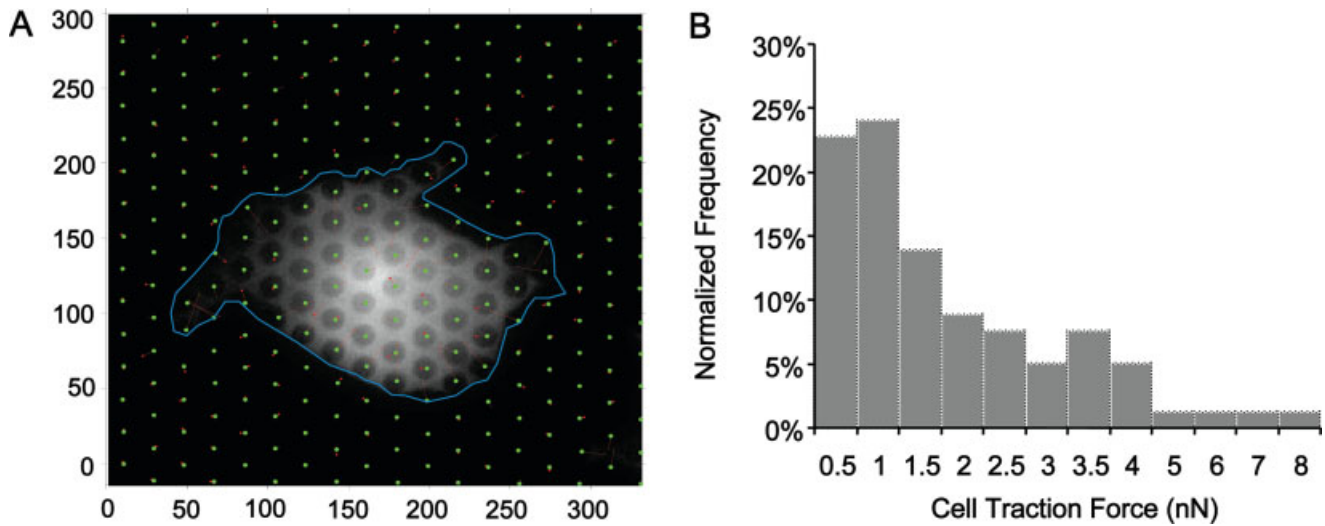


Fig. 7. CTFs determined using “*Top-Bottom*” method for a rat aorta SMC (see Fig. 3A). (A) CTF map. (B) Histogram of CTFs.

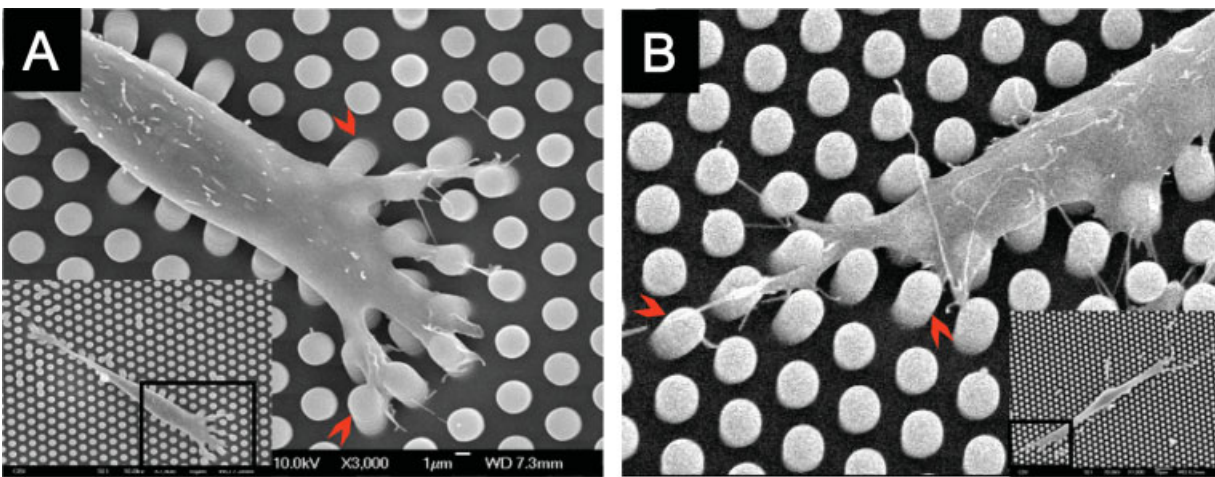


Fig. 8. SEM images of two types of fibroblasts adhered to MFSAs. (A) HSF; and (B) HPTF.

to be slightly smaller than their counterparts on smooth surfaces.

Detailed morphology of a spread cell on top of MFSAs was observed with SEM (Fig. 6C). Apparently, the cell established close contact with the microposts underneath and applied contractile force on the microposts. As a result, these microposts were bent, and the extent of bending (lateral deflection) at each micropost reflected the magnitude of CTF applied by the cell.

Cells grew equally well on the discrete MFSA and on continuum PDMS surface. No apparent difference between the numbers of HSFs on MFSAs and those on smooth PDMS substrates was observed at various culture times. Hence, these cells proliferated at a similar rate on the microposts and smooth surface, meaning that the

topography feature of microposts did not affect cell growth appreciably.

CTF Measurement Using MFSAs

The CTF measurement results from the aforementioned two image analysis methods were similar. A typical CTF map, obtained using “*Top-Bottom*” method for a SMC (Fig. 3), is shown in Fig. 7A. The displacement vectors as well as the cell boundary are superimposed to the top image of the MFSA to give a clear view of the direction and magnitude of CTFs. Apparently, the traction forces around the nucleus region of the cell are small, but they are large near the cell edge. As indicated from the histogram (Fig. 7B), the majority of CTFs applied on the microposts range from 1 to 4 nN, but

some can be as high as almost 8 nN at certain positions close to the edge. All CTFs are centripetal, which is consistent with the CTF measurement results using other methods such as traction force microscopy (TFM) [Beningo et al., 2002; Wang et al., 2002].

As another example of MFSA application, the CTFs produced by two types of fibroblasts, HSFs and HPTFs, were determined in this study. HSFs were found to bend the microposts to a much larger extent than HPTFs (Fig. 8). By comparing the deflection of microposts, it was found that the mean traction force of HSFs was more than twice that of HPTFs.

DISCUSSION

Fabrication of micropost arrays with a high density and aspect ratio (micropost height/micropost diameter) has gained increased interest in recent years [Petronis et al., 2003; Mohrdieck et al., 2005; Schmitz et al., 2005]. This is essential in order to extend the micropost array-based technique to measure CTFs for cells of smaller size and/or producing less force. These requirements also apply to fibroblasts which have spindle shape. HPTFs, for instance, typically have a width ranging from 5 to 20 μm , and are much smaller and more irregular at the two ends along the longitudinal direction. Micropost arrays with a periodicity larger than 10 μm are obviously not suitable for studying these cells because they cannot fully spread on the microposts; as a result, the morphology of cells is markedly distinctive from that of cells on a smooth surface, i.e., continuum substrate. We have estimated that even on a micropost array as dense as having a periodicity of 4 μm in our case, the size of fibroblast was $\sim 10\text{--}20\%$ smaller than that on a smooth surface. Probably due to the same reason, there was occasionally attachment of filopodia to the side wall of microposts, which complicated CTF computation. In order that the cells attach to and spread on the micropost array in a way similar to those on continuum substrate, the density of micropost must be high enough such that the distance between adjacent microposts is much smaller than the size of cell. Ideally, the CTF-sensing array should consist of posts far less than 1 μm apart so that cells grow on it as if they are growing on continuum surface, yet their CTFs can be quantified using these discrete post force sensors.

Besides the requirement of high density MFSAs for small cells, measuring small traction forces for cells like tendon fibroblasts also requires high-sensitivity MFSAs. One way to accomplish this is to make the microposts compliant enough such that they can be deflected to a larger extent in response to the small traction forces of the cell. Under this circumstance of large deflection, however, the small strain linear elastic Timo-

shenko beam solution is no longer valid for microposts. To circumvent this limitation, we used FEM analysis to compute the forces corresponding to different deflections of microposts. Our modeling results showed that although an approximately linear relationship between force and deflection holds true for small micropost deflection, which is consistent with small strain beam theory, force increases in a quadratic fashion when the lateral deflection is more than 0.5 μm for the microposts used in this work. Therefore, the linear algorithm is only applicable for small micropost displacement, which limits the application of micropost array in that arrays of various sizes of microposts might be needed for different types of cells. With the present application and interpretation of FEM results, however, the working range of force measurement is free from the limitation and has been markedly increased. As a result, CTFs, small or large, can be determined using microposts of the same geometry.

Nevertheless, while higher density of MFSA brings in a better resolution, it also creates a problem. Cells grown on top of these MFSAs become invisible under bright-field microscopy because of the intrinsic optical effect of microposts as individual waveguides. There is, therefore, a need to visualize the cells so that one can locate the target cells for related studies. Fluorescence labeling is an effective method for this purpose. In previous studies, cells were fixed and actin cytoskeleton fluorescently labeled such that cell boundaries could be defined [Tan et al., 2003; Lemmon et al., 2005]. However, this approach is only good when "end-point" data are to be collected; it is not applicable when dynamic monitoring of the traction forces is required. To this end, we used live-cell imaging technique in this work using a fluorescence calcium indicator, calcein-green AM ester (CGAM). Upon permeation into the cell, CGAM binds to intracellular Ca^{2+} ions and results in green fluorescence emission, which visualizes the cell with high contrast. A mere 5-min incubation of cells with CGAM is good enough to obtain clear cell image for deciding the cell boundary. We have found that prolonged incubation of cells with CGAM did not apparently affect CTF, which is also consistent with the findings using other methods such as cell traction force microscopy (CTFM) [Wang et al., 2002]. It should be noted, however, that although the cell border and major protrusions can be visualized by using CGAM, it is not clear yet whether the smaller/thinner cellular structures such as lamellipodia and filopodia can be equally well identified using this technique.

Existing micropost array-based CTF measurement methods generally assume that the microposts are perfectly arranged; therefore, the initial positions of microposts that contact with a cell can be interpolated through

the positions of microposts outside the cell region. In reality, however, the microposts possess some degree of initial deformation. Therefore, the interpolation algorithm cannot give the true deflection of microposts underneath a cell. This results in limited resolution of displacement measurement and subsequent force computation. None of the previous studies have addressed this issue. To improve the resolution, we developed two new image acquisition and processing methods. Both “*Before-After*” and “*Top-Bottom*” methods compare the real positions of microposts instead of interpolated ones, thereby bringing in further accuracy in determination of micropost displacements. An additional advantage of these two methods is that they do not require the microposts to be in a regular array; therefore, it is possible to use smaller “nanoposts” which are fabricated through non-lithographic techniques such as template-assisted nano-rod formation using anodic alumina template [Jessensky et al., 1998; Sander and Tan, 2003] and do not form a regular array. These features are important when higher density of micro-/nano-posts are employed because the smaller the force-sensing units, the easier they tend to deform and lose the regularity. Compared with “*Before-After*” method, the “*Top-Bottom*” method is slightly less accurate, but it can be conveniently used to measure CTFs of a cell at any time without the need to remove the cell; therefore, it is very suitable for dynamic traction force studies in which repeated CTF measurements during a certain time course are performed.

Using MFSA technology, it was clearly shown that HSFs produced much larger traction forces than HPTFs. This might indicate intrinsic differences in wound closure as well as scar tissue formation at the two types of source tissues, i.e., skin and tendon. Therefore, MFSA is a sensitive and useful technique that can aid in better understanding the cellular and molecular mechanisms of tissue wound healing. For example, MFSA technology may be used to measure CTFs of human fibroblasts in response to transforming growth factor- β 1 (TGF- β 1) and TGF- β 3 treatments at various dosages, as well as the dynamic development of CTFs under these treatments, and thus generate new insights into understanding of scar tissue formation.

Besides behaving as passive force-sensing units, the microposts in a MFSA can also be used as active devices for applying mechanical stimulation to cells. For example, microposts may function as microactuators that stimulate cells on MFSA under stretching. Tanaka et al. used microposts as bio-microactuators and demonstrated the feasibility to convert chemical energy of cardiomyocytes into mechanical energy [Tanaka et al., 2006].

In summary, we have developed an improved MFSA technology, including high density micropost array, new image acquisition and analysis methods for

determination of displacement of microposts, and a non-linear micropost displacement/deflection-force relationship for determination of CTFs. This MFSA technology can achieve a spatial resolution of 40 nm and a force sensitivity of 0.5 nN. As such, this technology will be a useful tool for a variety of biological applications, including studying the effect of cell shape and cytokines on CTFs and using CTF as a “biophysical marker” to detect cancerous cells.

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