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TECHNICAL INNOVATION

Three-dimensional microfiber devices that mimic physiological environments to probe cell mechanics and signaling†

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Many physiological systems are regulated by cells that alter their behavior in response to changes in their biochemical and mechanical environment. These cells experience this dynamic environment through an endogenous biomaterial matrix that transmits mechanical force and permits chemical exchange with the surrounding tissue. As a result, *in vitro* systems that mimic three-dimensional, *in vivo* cellular environments can enable experiments that reveal the nuanced interplay between biomechanics and physiology. Here we report the development of a minimal-profile, three-dimensional (MP3D) experimental microdevice that confines cells to a single focal plane, while allowing the precise application of mechanical displacement to cells and concomitant access to the cell membrane for perfusion with biochemical agonists. The MP3D device – an ordered microfiber scaffold erected on glass – provides a cellular environment that induces physiological cell morphologies. Small manipulations of the scaffold's microfibers allow attached cells to be mechanically probed. Due to the scaffold's minimal height profile, MP3D devices confine cells to a single focal plane, facilitating observation with conventional epifluorescent microscopy. When examining fibroblasts within MP3D devices, we observed robust cellular calcium responses to both a chemical stimulus as well as mechanical displacement of the cell membrane. The observed response differed significantly from previously reported, mechanically-induced calcium responses in the same cell type. Our findings demonstrate a key link between environment, cell morphology, mechanics, and intracellular signal transduction. We anticipate that this device will broadly impact research in fields including biomaterials, tissue engineering, and biophysics.

Introduction

Although several approaches have been used to investigate cellular responses to mechanical stimuli (*i.e.*, mechanotransduction),[1–4] we chose to develop a fiber-based device to investigate mechanically-induced intracellular calcium signaling because calcium plays a critical role in cellular processes ranging from the rapid release of synaptic vesicles in neural firing, to the significantly slower phenotypic remodeling and gene transcription observed in cardiac hypertrophy.[5,6] Furthermore, it can be robustly activated by both mechanical stimuli and chemical agonists.[1,4,5,7–9] In order to understand the integration of calcium signaling resulting from mechanical and chemical stimuli, we attempted to decouple these signals by comparing intracellular responses to each stimulus applied at separate times in a single cell. Previous studies in chemically-induced calcium signaling significantly outnumber those studies investigating mechanics. As a result, we developed a new micromechanical, fiber-based device capable of creating novel mechanical microenvironments to enable the observation of calcium signaling following mechanical stimulus.

For such a device to create physiologically relevant environments, it would ideally be tailored to reproduce the specific *in vivo* mechanical constraints a cell experiences.[10] Although various approaches for mechanically probing cells *in vitro* do exist, many of these current approaches rely on 2D-constrained planar glass or polymer surfaces, such as those used with optical tweezers,

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magnetic needles, or flexible substrates.[4,7,11] However, physiological cell environments are almost always three-dimensional. In these environments, cells often couple their intracellular cytoskeleton to an extracellular matrix (ECM) of biopolymers using transmembrane protein assemblies called focal adhesion complexes (FACs), which play an important role in cell signaling and phenotype regulation.[1,8,10,19]

One example of the importance of 3D microenvironments can be observed in fibroblasts. Fibroblasts adopt a stellate morphology (cellular processes extending radially from the soma in a star-like shape) in tissue or *in vitro* ECM hydrogels, but dramatically spread and form a laminar structure with a raised nuclear bulge when cultured on 2D substrate.[1] Previously, it was shown that NIH 3T3 fibroblasts assume physiological morphologies when cultured within a micron-gap, polyacrylamide bilayer *in vitro*. Cells within bilayers form both dorsal (on the cell's upper surface) FACs as well as ventral (on the cell's lower surface) FACs and adopt characteristic stellate morphologies.[1]

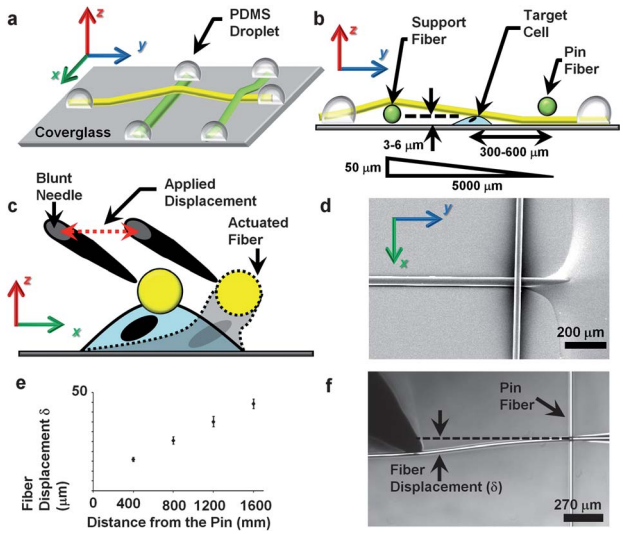

Fig. 1 MP3D devices for probing cells with mechanical and chemical stimuli. (a) Polypropylene scaffolds were erected on glass, consisting of microfibers aligned in a crosshair pattern with an *actuated fiber* (yellow) stretched over a central *support fiber* (green) and held against the glass by a *pin fiber* (green). PDMS droplets were used to fix the polymers in place. The actuated fiber can be mechanically manipulated with a micromanipulator-mounted needle. Cells that attach to the upper fiber can then be deformed. Three principal axes are provided for orientation. (b) Side view of the MP3D device showing relative geometries of its gap size. Here, a 3 to 6 μm gap for dorsal attachment is specified based on the cell height of NIH 3T3s. (c) An alternate side view showing the process of cell deformation. A blunt tipped needle, attached to a micromanipulator (not shown), displaces the actuated fiber, thereby deforming the cell. (d) An SEM image of an MP3D device showing the actuated (horizontal) fiber, the pin fiber, and the PDMS droplet prior to cell culture. (e) Application of a large (46.3 + 2.3 μm) displacement at 1650 μm distal to the pin elicited a range of displacements from the original position, measured at points 400, 800, 1200 and 1600 μm away from the pin fiber. Recorded displacements from the original fiber position are plotted for a fiber for each position distal to the pin fiber. Mean + Standard Error (S.E.), n = 3. (f) The mechanical fiber of a MP3D device is actuated by a micromanipulator. While only small displacements were needed for calcium experiments, even with relatively large displacements, several times (*e.g.* 70 μm) those realistically experienced by cells *in vivo*, the device behaved elastically, with complete recovery to the original fiber position.

Unfortunately, 3D *in vitro* experimental environments have their own drawbacks. First, for mechanical experiments, it is extremely challenging to decouple compression and tension when mechanically stimulating a cell encapsulated in ECM hydrogels. Second, cells in these systems are typically not amenable to observation with standard epifluorescent microscopy, instead requiring confocal systems, as their structures and processes extend out of a single focal plane. Third, one of the most common, conventional 3D approaches – hydrogel encapsulation – limits the quick exchange of chemicals in the extracellular environment, making experiments that require temporally-precise, chemical and mechanical stimulation of single cells quite challenging.

Motivated by these challenges, we developed the MP3D device to quickly and precisely provide chemical and mechanical stimuli to cells in 3D environments. The device provides realistic dorsal and ventral boundary conditions and also allows for carefully prescribed mechanical perturbations, the results of which can be observed and recorded. Furthermore, because cells are not encapsulated by the MP3D scaffold, the remaining exposed cell membrane can be perfused with biochemical agonists, thus allowing for the application of both mechanical and chemical stimuli to fibroblasts assuming physiological morphologies. The

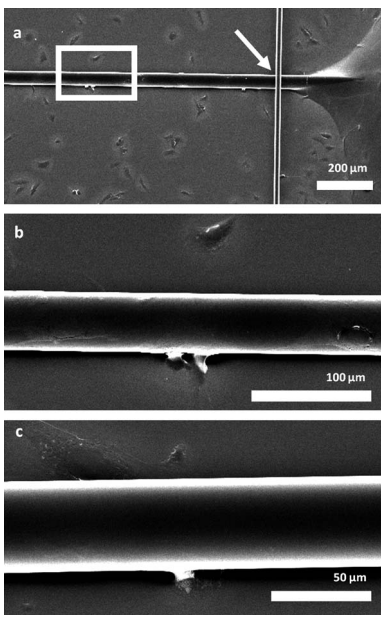

Fig. 2 MP3D devices induce physiological morphologies. (a) An SEM image of an MP3D device with NIH 3T3s cultured on its surface. Cells were allowed to migrate and attach to the fiber over a 24 to 48 h period. The horizontal fiber, which would be actuated in live-cell experiments, is below the vertical fiber, which serves to pin the horizontal fiber against the glass at the point indicated (arrow). The gap varies linearly from 0 at the pin to a maximum height at the support fiber (not shown). (b) Increased magnification of the indicated (box) region shows cells forming attachments to the horizontal fiber and adopting physiological morphologies. (C) A cell (foreground) extends itself to make contact with the fiber, whereas another cell (background, left) not making contact with the actuating fiber has a planar morphology with cell topology revealed by organelles.

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MP3D device includes 3D environmental features, enables simultaneous long term culturing and imaging capabilities, and, due to their minimal feature profile, allows cells to remain in a single focal plane, facilitating observation with conventional epifluorescent microscopy.

Each MP3D device consists of an ordered, fibronectin-coated stack of polymer microfibers on a coverglass (Fig. 1) and provides dorsal-ventral separation ranging from 0 to 50 μm (Fig. 1b). This separation can be controlled through adjustments in fiber size and positioning (ESI, Figs. S1 and S2). Separations of 3 to 6 μm have been shown to be particularly efficient at forming dorsal attachments for fibroblasts;[1] thus, this gap size is included in the separation range for the MP3D device described here, although the range could be altered for potential experiments with other cell types. To impose mechanical displacement on cells, the MP3D upper fiber can be actuated parallel to the plane of the coverglass by a micro-manipulated needle tip, potentially creating a large range of elastic displacements (Fig. 1). These displacements are also affected by the fiber diameter, a parameter that can be controlled in the fabrication process (ESI, Fig. S2). For experiments in living cells, only significantly smaller, physiological displacements (10–20 μm), were necessary to evoke an intracellular calcium response.

Materials and methods

MP3D device construction

Minimal-Profile-3D devices were constructed by drawing melted polypropylene manually at ambient temperature to form microfibers (25 to 50 μm diameter) and stacked perpendicularly (2 fibers high) on #1 borosilicate glass coverglass (22 mm × 40 mm), providing a variable gap between the glass surface and the uppermost fiber (Fig. 1). Thermally-cured PDMS (Sylgard 184, Dow Corning) droplets were used to secure fibers in grid patterns. MP3D devices were sterilized in 70% ethanol, rinsed in phosphate buffered saline (PBS), and incubated for 1 h in 20 mg mL^{-1} fibronectin to promote cell attachment.[12] This process is further detailed in Fig. S1 (ESI). Additionally, the ESI contains detailed methods including descriptions of cell-culture, live-cell fluorescent imaging, electron microscopy, and confocal microscopy techniques used here.

Results and discussion

NIH 3T3 fibroblasts grew robustly on MP3D devices in a manner similar to typical cell culture on fibronectin coated surfaces (ESI, Fig. S2). To confirm that cells were indeed extending processes to the device's upper fiber, cells seeded on MP3D devices were imaged using scanning electron microscopy (SEM). SEM images were collected as previously described.[13] Cells adopted a more stellate morphology, and consistently formed attachments on the upper fiber (Fig. 2). In order to confirm that fibroblasts were establishing a continuous, three-dimensional mechanical link from the dorsal to ventral surface, we visualized the distribution of the actin cytoskeleton in cells cultured in the MP3D system and on glass alone (Fig. 3). We observed continuous actin filaments from the ventral to dorsal surface in cells making contact with the MP3D system. In cells cultured on glass alone, actin cytoskeletons were only significantly present near the ventral surface immediately proximate to the substrate in a two-dimensional planar pattern. We concluded

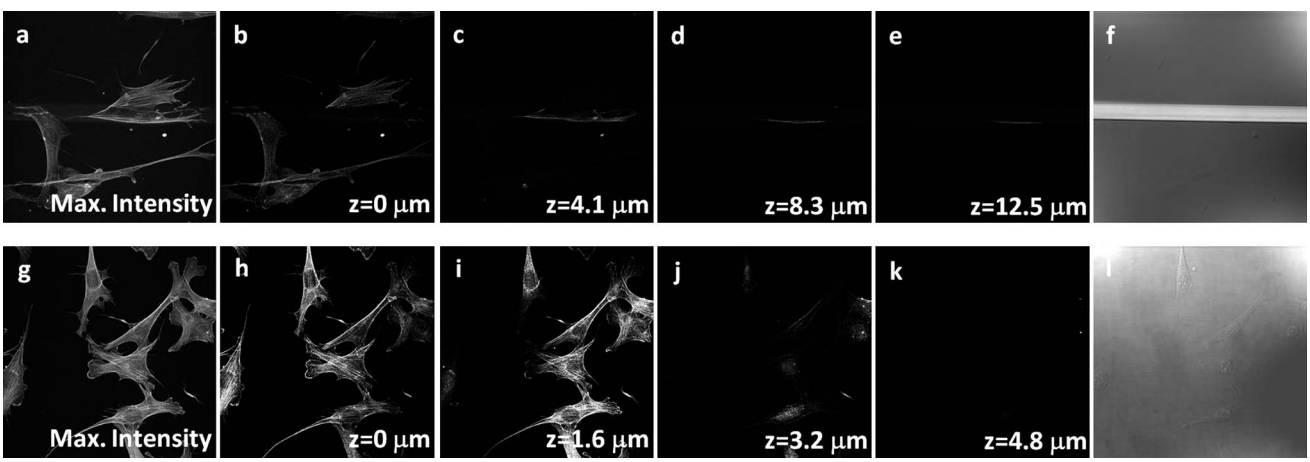

Fig. 3 Confocal images of NIH 3T3 actin cytoskeletons in cells cultured within the MP3D system and on glass alone. (a) Maximum intensity projections of actin in a confocal image stack of cells grown in the MP3D system also available as Supplementary Video 1. (b–e) Actin distribution at different heights from glass substrate to the upper fiber. (e) Actin distribution near the diameter cross-section of the actuated fiber on the dorsal (upper) surface of the cell. (f) Transmitted light image of the diameter cross-section of the actuated fiber. As the focal plane moves away from the glass surface toward the fiber in the MP3D system, the cytoskeleton of a cell in contact with the system follows the curvature of the upper, actuated fiber as cells attach ventrally to the glass and dorsally to the polypropylene fiber (*e.g.*, at the highest shown plane of 12.5 μm in (e), actin can still be visualized). (g) Maximum intensity projections of actin in a confocal image stack of cells grown on glass alone also available as Supplementary Video 2. (h–k) Actin distribution at different heights from the substrate (glass) surface upward. (i) Transmitted light image of the cells on glass. As we examine focal plane farther away from (above) the glass surface, cytoskeletal proteins quickly vanish in cells on glass alone in comparison to cells that interact with the MP3D system (*e.g.*, at the highest shown plane of 4.8 μm in (k), actin is no longer present whereas at the highest shown MP3D plane of 12.5 μm in (e), actin can still be visualized). Actin was stained as described in the ESI. With the exception of contrast stretching and background subtraction, these data are unmodified with no other image processing applied. All images are 246 × 246 μm.

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that these three-dimensional cellular attachments to both ventral substrate and dorsal fiber would be sufficiently robust to support mechanical strain resulting from fiber displacement.

We compared the response to mechanical and chemical stimulus in the same single NIH 3T3 fibroblast, by monitoring calcium transients resulting from membrane displacement applied using the MP3D device, followed by application of chemical stimulus. Intracellular calcium can be measured using a range of synthetic, fluorescent dyes or recombinant, GFP-based probes; we chose the widely-used Fluo-4 dye.[14] Chemical stimuli typically produce an observable elevation in fluorescent probe intensity that can be directly correlated with cytosolic $[Ca^{2+}]$.[14] This elevation often produces a visible "spike" in the calcium signal waveform. In our studies, we chose to first apply mechanical stimulus and then apply traditional chemical stimulus (Fig. 4), to assure that the mechanical stimulus had not damaged the cell and destroyed canonical calcium handling pathways and machinery (*e.g.*, pumps that extrude calcium from the cytosol following stimulus).

Calcium responses resulting from traditional chemical stimulation were induced by applying adenosine triphosphate (ATP), a robust agonist of purinergic (P2Y) receptors that couple directly to ER calcium stores through the canonical $G_{\alpha,q/11}/IP_3$ second-messenger cascade and has been shown to mobilize calcium in fibroblasts.[15] We found that stretching the upper membrane of the cell created a spike in intracellular $[Ca^{2+}]$ similar to the waveform observed in the following chemical stimulation (Fig. 4 and Supplementary Video 3). This data represents one of the few times $[Ca^{2+}]$ time courses resulting from both chemical agonists and mechanical tension applied to cell membrane attachments have been recorded in a single cell. Additionally, the data shows that cells can produce a similar response to both mechanical and chemical stimuli.

Furthermore, the mechanically-induced calcium signaling profile we observed is significantly different from that observed by Wang and colleagues in experiments with NIH 3T3 fibroblasts cultured on planar, soft substrates.[4] In their experiments, cells adopted planar, 2D morphologies and experienced mechanical stimulus through substratum stretching. Following stimulus, they showed an increase in cytosolic calcium even 10 min after the initial stimulus. They observed calcium responses that significantly differ from those in our experiments, in which calcium rapidly spiked, and was quickly extruded in a manner similar to the chemically-induced response in the same cell. This type of fast on-off response (*i.e.*, ranging in the tens of seconds) is a hallmark of the calcium-handling machinery.[5,16,17] As a result, the difference between our studies suggests the involvement of distinct calcium pathways, potentially the result of phenotypic remodeling motivated by different spatial and mechanical environments.

In the future, these different modes of calcium mechanotransduction may potentially be probed by extending the MP3D technique with inspiration from recent work by the Yamada group in one-dimensional motility assays using fiber-like substrate patterning.[18] These investigators were able to control motility speed as a function of pattern thickness with cells tending toward moving faster as smaller fiber diameters. As we noted above, we can control the upper fiber diameter (ESI, Fig. S2), and thus testing calcium across a range of fiber diameters in our MP3D device would be intriguing, as devices with smaller fibers could potentially target cells with less temporally well-established connections to our system.

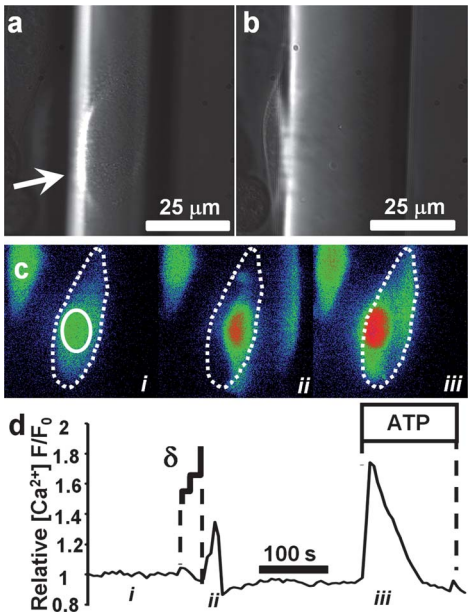

Fig. 4 MP3D devices induce robust calcium responses. (a) Differential interference contrast (DIC) image of a single cell located (white arrow) directly under the upper fiber of the MP3D device. The image's focal plane occurs just above the coverglass upon which cells are grown. (b) A DIC image with the image focal plane focused on the upper fiber cross-section. To confirm attachment to the upper fiber for cell selection prior to experiments, the focal plane is slowly actuated vertically from the coverglass surface to the fiber cross-section to verify that part of the cell remains in focus and follows the curvature of the fiber. (c) Pseudo-color fluorescent images showing the cell prior to the application of displacement (i), immediately after maximum displacement (ii), and after treatment with 100 μM ATP, a purinergic agonist (iii). The solid white circle indicates the region of interest (ROI) for fluorescence intensity measurement. The dotted white circle indicates the cell perimeter prior to stretching. (d) Time course of the normalized fluorescent intensity (F/F_0) in the indicated ROI (white circle, c). Points (i, ii, and iii) corresponding to the images in Fig. 4C are noted. The total displacement (δ) provided was ~18 μm, reached over three sequential steps lasting 20 s each. A calcium spike was observed upon the application of the third displacement step. Calcium signal time course shown here is representative of n = 4 cells in 3 experiments. The entire image series is provided (Supplementary Video 3).

Conclusion

The novel chip-based experimental system we describe here enables the identification of new biological phenomena by allowing for careful control of the extracellular chemical and mechanical milieu. The MP3D device capitalizes on the flexibility of polymeric substances to create microstructural environments of prescribed geometries that can be mechanically manipulated and perfused with biochemicals. Additionally, the minimal-profile features of these microstructures allow for cellular experiments in a 3D environment with standard epifluorescent microscopy. As a result, the device can be fully integrated with

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existing technology to enable a broad range of new approaches for analyzing cell-scale phenomena. Finally, the different response in calcium signaling we observed with the MP3D device, compared to previous studies, highlights the importance of microscale devices designed to replicate three dimensional features of *in vivo* mechanical interfaces. It also underscores the importance of employing different *in vitro* experimental systems when attempting to draw a complete picture mechano-transduction in cells. To that end, although we designed the MP3D system to interface with the most standard epifluorescent, live-cell microscope setups, in order to extend the usefulness of our approach, the throughput of the device could be expanded to allow the analysis of multiple cells in a variety of chemical and mechanical conditions. We envision the development of a multi-well insert that allows consistent fiber architectures to be placed within each well of a plate. A reuseable array of microactuators could then be interfaced with the insert during experiments. Combining actuator control with fluidic perfusion control and an X-Y servo-driven microscope stage would allow software-level control of high-throughput chemomechanical assays using the MP3D fiber system. In the future, we expect the MP3D device we describe here will be deployed and adapted to analyze phenomena ranging from signal transduction in fibrous biomaterial matrices to biophysical modeling of cellular responses to mechanical stimuli.

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