Uniaxial cell stretching device for live-cell imaging of mechanosensitive cellular functions

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External mechanical stretch plays an important role in regulating cellular behaviors through intracellular mechanosensitive and mechanotransductive machineries such as the F-actin cytoskeleton (CSK) structures and focal adhesions (FAs) anchoring the F-actin CSK to the extracellular environment. Studying the mechanoresponsive behaviors of the F-actin CSK and FAs in response to cell stretch has great importance for further understanding mechanotransduction and mechanobiology. In this work, we developed a novel cell stretching device combining dynamic directional cell stretch with *in situ* subcellular live-cell imaging. Using a cam and follower mechanism and applying a standard mathematical model for cam design, we generated different dynamic stretch outputs. By examining stretch-mediated FA dynamics under step-function static stretch and the realignment of cell morphology and the F-actin CSK under cyclic stretch, we demonstrated successful applications of our cell stretching device for mechanobiology studies where external stretch plays an important role in regulating subcellular molecular dynamics and cellular phenotypes. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4832977>]

I. INTRODUCTION

In the last decade, mechanical signals in the cellular microenvironment, e.g., extracellular matrix (ECM) stiffness, ECM micro/nanotopography, and external mechanical forces, have been demonstrated to be of great importance in regulating cellular behaviors from single cellular to tissue scales, contributing to human development and diseases.^{1–6} As a stimulus of significant physiological relevance, mechanical stretch has been well known for its important role in regulating tissue morphogenesis, physiological homeostasis, and pathogenic processes in endothelial, epithelial, and smooth muscle cells.^{7–12} *In vitro* studies of cellular behaviors under external dynamic directional stretch that mimics *in vivo* conditions have helped reveal novel mechanobiological phenomena spanning from cellular phenotypes such as morphogenesis,^{13–16} proliferation,^{17,18} differentiation,^{19–21} and inflammation^{22,23} to gene expression profiles.^{24–26} In addition, recent progress in mechanobiology has led to the discovery of a range of molecular mechanosensors, including focal adhesions (FAs),^{27–29} clathrin coated pits (CCPs),³⁰ caveolae³¹ and ion channels.^{28,32} Studying the mechanosensitive subcellular dynamics of these molecular mechanosensors under external stretch can help elucidate the underlying bio-

physics of these force-dependent molecular systems and thus provide profound insights in revealing the mechanotransduction mechanisms. To facilitate the study of mechanotransductive processes in living cells, a cell stretching device capable of both versatile dynamic directional stretch and *in situ* imaging of subcellular molecular dynamics in living cells could be critical for studies aimed at a deeper understanding of the fundamental connection between mechanical stretch and cellular physiology.

Numerous cell stretching devices have been developed in previous studies and applied to explore cellular behaviors in response to controlled external stretch.^{9,14,17,21,33–40} However, many of these implementations have significant limitations. With the exception of several state-of-the-art devices, such as the commercial Flexcell systems, which rely heavily on sophisticated pneumatic control systems for regulating air pressure, the regulation on the dynamic stretch is not demonstrated for most vacuum-driven cell stretchers.^{34,37,38} For mechanically driven cell stretching devices, on the other hand, many of them are not compatible with subcellular live-cell imaging *in situ*, which is critically required for studying the dynamics of molecular mechanosensors.^{9,33} So far, only a few studies have demonstrated cell stretching devices that are capable of real-time subcellular live-cell imaging, but all with limitations in applying dynamic, especially cyclic, directional stretch to cells.^{34–37,39} Therefore, it is necessary to develop a novel device that can achieve both simple, versatile design of dynamic directional stretch while accessible to subcellular live-cell imaging *in situ*.

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In this study, by implementing a cam and follower mechanism for the first time in the development of the cell stretching device, we show that an easy and versatile design strategy can be achieved by using a standard mathematical model that enables the rational design of cam geometry and dimensions for dynamic stretch of desired waveforms. Through microscope-specific customization and by using thin elastomeric polydimethylsiloxane (PDMS) membrane fabricated by spin-coating, we demonstrated the capability of our device by examining subcellular FA dynamics under step-function

directional stretch and tracking the realignment of cell morphology and the F-actin CSK structure under long-term cyclic directional stretch.

II. DEVICE DESIGN AND SPECIFICATIONS

A. Device design and implementation

The design of our device is illustrated by the CAD model shown in Figure 1(a). The structural frame of the device was

FIG. 1. Design and implementation of the cell stretching device. (a) The CAD model of the device. (b) Design and assembly of the membrane assembly and cell culture holder. (c) Design of the clamping module and mounting of detached membrane assembly with clamps onto the two L-shaped plates. (d) Schematic of the membrane stretching displacement generated by the cam and follower mechanism. (e) Picture of the implementation of the cell stretching device. (Inset) A zoom-in image of the cam and follower mechanism.

customized to fit as a stage insert on the motorized stage with the motor components of the device off to the side to avoid interference with the light path of an inverted fluorescence microscope. The functionalities of the device were performed by the symphony of three modules: (1) the clamping module, consisting of two L-shaped plates (or L-plates) – one was fixed to the basal plate and the other was free, firmly anchored a thin PDMS membrane with clamps and positioned it under the microscope light path; the clamping module was connected to the stretching module via the free L-shaped plate; (2) the stretching module, which implemented a cam and follower mechanism, generated translational movement of the L-plate-shaft-follower linkage under the guidance of linear bearings, and thus stretched the PDMS membrane in a directional manner; and (3) the motor module, controlled by a customized electrical control board and built-in programs, regulated the rotation of the cam and thus the movement of the follower and shaft as well as the stretch of the membrane.

To facilitate cell seeding, culture, and sample preparation, we developed a holder for assembling the PDMS membrane before seeding and culturing cells on the PDMS membrane. As shown in Figure 1(b), a free-standing PDMS membrane was assembled between two separated sets of clamps on the base of the holder. A small cell culture well cut out of a PDMS block was then affixed onto the membrane using silicone vacuum grease. This modular membrane assembly and cell culture holder uncoupled sample preparations from cell stretching experiments.⁴¹ Before cell stretching experiments, the membrane assembly with two clamps was detached from the holder before mounted onto the base of the two L-plates of the clamping module.⁴¹ Two adjustment screws were implemented to adjust the height of the L-plates to accommodate microscope-specific altitude of the objective stage while keeping the membrane in a flat horizontal configuration.⁴¹ By using PDMS membranes of different widths, our device was able to perform either single or parallel tests during one experiment.⁴¹ Figure 1(d) illustrates the working principle of the stretching module. By modulating the rotation and dimension of the cam, it provided a straightforward, yet robust way to control the translational displacement of the free L-plate, and thus the dynamic stretch of the membrane.⁴¹

B. Cam design and waveform generation for cyclic stretch

A significant advantage of the cam and follower mechanism was that it enabled an easy and robust method to modulate the waveform of dynamic stretch within the membrane. By rationally designing the geometry and dimension of the cam using a standard mathematical model, we demonstrated the design of three different cams generating sinusoidal waveforms with stretch amplitudes of 5%, 10%, and 15%, respectively.

As shown in Figure 2(a), the contour of the cam was represented by a function, $r(\theta)$, in a polar coordinate system, describing the variation of the cam radius with its rotation. In principle, $r(\theta)$ could be any continuous periodic function with a period of 2π or 2π divided by an integer. Since the movement of the follower and the shaft faithfully followed the contour of the cam, their translational displacement as well as the stretch of the membrane would observe the same waveform as $r(\theta)$. Therefore, to generate a sinusoidal stretch waveform, the mathematical model dictated

$$r(\theta) = \frac{1}{2}(r_1 + r_2) + \frac{1}{2}(r_1 - r_2) \times \sin(\theta), \quad (1)$$

where r_1 and r_2 were the maximum and the minimum of $r(\theta)$, respectively. The stretch ratio, λ , could be expressed explicitly as

$$\lambda = \frac{r_1 - r_2}{L_0}, \quad (2)$$

where L_0 was the resting length of the PDMS membrane without stretch. Combining Eqs. (1) and (2), we obtained the master equation for designing the cam

$$r(\theta) = \left(r_1 - \frac{1}{2}\lambda L_0\right) + \frac{1}{2}\lambda L_0 \times \sin(\theta). \quad (3)$$

In our design, the dimensions for the cam were chosen as following: $r_1 = 25.4$ mm, $L_0 = 40$ mm. Using Eq. (3) and $\lambda = 5\%$, 10%, and 15%, we obtained the design contours for three different cams (Figure 2(a)), which could generate sinusoidal directional stretch of 5%, 10%, and 15% for the PDMS membrane (Figure 2(b)), respectively. The cams were easily

FIG. 2. Design of cam. (a) Schematic of the contours of three different cams in a polar coordinate system. (b) The waveform generated by the three cams in one cycle of rotation.

interchangeable for achieving the desired stretching amplitude using our cell stretching device.

In addition to the rational design of cam geometry and dimension, one could acquire additional control on the waveform of the stretch by programming the rotation speed of the motor.⁴¹ In our tests, the device could achieve cyclic dynamic stretch with the stretch frequency from 0 Hz up to 10 Hz, covering the physiologically relevant range of frequency.

C. Effect of membrane size on stretch uniformity

Since our device was designed to accommodate both single test and parallel tests in one experiment,⁴¹ it became critical to validate that the magnitude as well as the uniformity of stretch within the PDMS membrane was not significantly affected by the width of the membrane. In this study, we built finite element (FE) models (ABAQUS 6.9.1) for PDMS membranes of different widths under directional stretch. The PDMS membrane was considered as a two-dimensional (2D) thin layer with a thickness of 200 μm and made of a neo-Hookean material with the Young's modulus of 2.5 MPa and Poisson's ratio of 0.42. It was fixed on the left edge while the right edge was subject to displacement along the x -axis resulting in 15% nominal stretch.

FE simulation results of the logarithmic tensile strain field within the membranes were shown in Figure 3. For both longitudinal and transverse tensile strain fields, it was clear that although membranes of different widths exhibited varying patterns of the strain fields, owing to the membrane size

effect on the boundary conditions, the central part of the membrane, on which cells were to be cultured, underwent uniform stretch. Furthermore, we quantified the magnitude and uniformity of the stretch by plotting the longitudinal and transverse stretch ratio along a path of fixed length cutting through the center of the membrane (Figures 3(c), 3(f), and 3(i)). It is important to note that even though quite uniform, a transverse stretch (-6%) was simultaneously generated along with the longitudinal stretch (15%) due to the free lateral boundaries and the high Poisson's ratio of PDMS. Therefore, our device generated uniform "elongation," instead of uniaxial stretch, within the membrane, a phenomenon common to existing uniaxial cell stretching devices.

D. Calibrations

To experimentally determine the uniformity of membrane stretch generated by our device, we printed a 1 cm $\times$ 1 cm array of microscale circular islands of fluorescently labeled bovine serum albumin (BSA, conjugated with Alexa Fluor-647) onto the PDMS membrane using microcontact printing.^{41,42} Before stretch, the diameter of these circular islands was 32 μm with the island center-to-center distance of 60 μm . To examine the uniformity of the stretch within the cell-culture region, we selected eight representative locations (Figure 4(a)) and recorded the fluorescent circle array both before and after a step stretch was applied (Figure 4(b)). Using the fluorescent circle array as fiducial markers, we calculated the longitudinal and transverse stretch ratio at the

FIG. 3. Finite element (FE) simulations of membrane size effect on the uniformity of the stretch. Tensile strain fields (a) and (b) and extracted stretch ratio (c) within a 40 mm $\times$ 40 mm PDMS membrane. Tensile strain fields (d) and (e) and extracted stretch ratio (f) within a 40 mm $\times$ 20 mm PDMS membrane. Tensile strain fields (g) and (h) and extracted stretch ratio (i) within a 40 mm $\times$ 14 mm PDMS membrane. The data plot path is 14 mm along the middle of membrane.

FIG. 4. Experimental validation of the uniformity of membrane stretching. (a) Schematic of the representative locations selected for characterizing the deformation field throughout the cell-culture region on the membrane. (b) Fluorescent images of micropatterned circular islands at different locations, before and after stretch. (c) Distribution of longitudinal and transverse stretch ratio at different locations. Error bar: standard deviation. Note that the slightly less longitudinal strain (by $<2\%$) from the designed 15% stretch was due to the fact that the starting point of cam rotation was not exactly at the lowest stretch.

different locations using ImageJ.⁴¹ As illustrated in Figure 4(c), the stretch along both the longitudinal and transverse directions was uniform throughout the cell-culture region in the center of the PDMS membrane. It is also important to note that our calibration results agreed well with the theoretical results from our FE simulations (Figure 3), validating the usefulness of our FE models for the rational design of the device.

III. MATERIALS AND METHODS

A. Fabrication of PDMS membrane

The thin PDMS membrane was fabricated by spin-coating. In brief, Sylgard 184 PDMS (Dow Corning, Midland, MI, USA) was mixed at a base/curing agent ratio of 10:1 and degassed. PDMS was then spin-coated onto either the top or bottom surface of a petri dish using a spin-coater (Laurell Technologies Co., North Wales, PA, USA) at 500 rpm for 30 s. Such spin-coating method generated PDMS membranes about 200 μm thick, as measured by a profilometer.

B. Cell culture

Rat embryo fibroblast cell line REF-52 stably transfected with YFP-paxillin was cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 $\mu\text{g}/\text{ml}$ glutamine, at 37 $^{\circ}\text{C}$ and 5% CO_2 .

The PDMS cell culture wells were manually cut out from a cured PDMS block using a razor blade and a hole with a diameter of 0.5 inch was punched in the middle, within which

cells were cultured. The PDMS well was affixed to the PDMS membrane using silicone vacuum grease (Dow Corning, Midland, MI, USA), which was biocompatible and hydrophobic, and provided minimal friction between the well and the membrane underneath during membrane stretching.

Cells were re-suspended from tissue culture plates using 0.25% trypsin-EDTA and seeded at 4000 cells per well for all cell stretching experiments. In order to culture cells on the PDMS membrane, the PDMS surface was functionalized with fibronectin using microcontact printing. In brief, a PDMS stamp was inked with 50 $\mu\text{g}/\text{ml}$ fibronectin (from human plasma, 0.1% solution, Sigma-Aldrich) for 1 h, then blown dry with nitrogen gas and brought into conformal contact with UV ozone-activated PDMS membrane for 30 s.

C. Fixation and immunocytochemistry

Cells were fixed using 4% paraformaldehyde (diluted from 16% solution of paraformaldehyde, methanol free, Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for 20 min. Cell membrane permeabilization and blocking of non-specific binding were achieved using 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in 10% goat serum (Life Technologies, Carlsbad, CA, USA) for 1 h. F-actin was labeled using Alexa Fluor-555 Phalloidin (Life Technologies, Carlsbad, CA, USA), and nucleus counterstaining was achieved using DAPI (Life Technologies, Carlsbad, CA, USA). The central area of the PDMS membrane on which cells were cultured was carefully cut out using a razor blade and mounted onto a glass slide with Slowfade Gold

anti-fading reagent (Life Technologies, Carlsbad, CA, USA) and sealed with a glass coverslip before imaging.

D. Live-cell imaging

For subcellular *in situ* imaging of FA dynamics under directional stretch, inverted epifluorescence microscope (Zeiss Axio Observer Z1, Carl Zeiss Microimaging, Thornwood, NY, USA) equipped with a monochrome charge-coupled device (CCD) camera (AxioCam, Carl Zeiss MicroImaging) and a 40× EC Plan-Neofluar objective (NA 0.75, Ph2, Carl Zeiss MicroImaging) was used. Experiments tracking cell morphological realignment under cyclic stretch were performed using a Nikon Eclipse Ti microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with sCMOS camera (C11446 Orca Flash 4.0; Hamamatsu) and a 10× objective (NA 0.30). Because of thinning of PDMS membrane under stretch, we manually re-adjusted focus before acquiring cell images after the stretch was applied. Alternatively, one might also use advanced autofocus system to mitigate such issue of re-focusing. The growth medium was replaced with phenol red-free imaging medium buffered with 2% HEPES before cell stretching experiments, and the medium was replenished every 30 min for long-term culture over 3 h on the microscope stage. The environment for cells imaged on the microscope stage was kept at 37 °C by an ASI 400 air stream incubator (NEVTEK, Williamsville, VA, USA). All images were analyzed using software ImageJ (NIH).

IV. RESULTS

A. Force-mediated focal adhesion dynamics under step-function directional stretch

Several subcellular molecular machineries have been recently identified as critical mechanosensors for cellular mechanotransduction.^{27–32} In order to study the biophysics underlying force-dependent dynamics of these molecular systems, it calls for *in situ* subcellular live-cell imaging with simultaneous applications of external force to the cell. Here, we demonstrated the utility of our cell stretching device by studying force-mediated FA dynamics under directional stretch.

For the sake of simplicity, we applied a nominal step-function stretch of 15% to cultured REF-52 cells expressing YFP-paxillin and acquired live-cell fluorescence images of FAs before and 1 min after the onset of stretch. As illustrated in Figure 5, fluorescence intensity of FAs containing YFP-paxillin increased significantly after stretch was applied, suggesting a rapid FA reinforcement under external force. Our observation is in support of the theory of force-mediated FA dynamics⁴³ and is also consistent with results reported by others.³⁵ Given its unique capability for external stretch and subcellular live-cell imaging, our device provided a useful platform for studying mechanosensitive subcellular FA dynamics as a critical component of cellular mechanotransduction.

FIG. 5. Fluorescent live-cell images of YFP-paxillin (a) and zoom-in images (b) and (c) of focal adhesions before and after a step-function stretch (inset) of 15% was applied, demonstrating a rapid force-mediated focal adhesion assembly. Scale bar: 20 μm .

B. Realignment of cell morphology and F-actin cytoskeleton under cyclic directional stretch

Cyclic directional stretch has been well known as an important regulator in vascular physiology and homeostasis. Diseases in cardiovascular blood vessel are often found correlated with abnormal mechanical stretch applied to the endothelium.^{7,8,10} Imaging cellular behaviors under cyclic stretch could provide important insights into the temporal evolution of mechanoresponsive cellular phenotypes such as morphology and proliferation. Here, we applied our device to study the realignment of cell morphology and F-actin CSK under cyclic directional stretch, which is a fundamental process sharing common features with endothelial morphogenesis *in vivo*.¹⁰

Being compatible with *in situ* live-cell imaging, our device allowed us to apply 2 Hz cyclic directional stretch of a sinusoidal waveform, with the nominal stretch ratio of 15%, to cultured cells for more than 3 h on the microscope stage. This allowed us to track the temporal dynamics of cell morphology either across the entire cell-culture area or in specific regions of interest, even down to a single-cell level, as demonstrated in Figure 6(a). From the phase contrast images, it is clear that cells realigned the major axis of their cell morphology as well as actin stress fibers (Figure 6(c)), to become in perpendicular with external stretch. A more detailed examination revealed that cells first rounded up in the first 60 min after the application of cyclic stretch, before spread out again with the new major axis of their cell morphology aligned perpendicular to the direction of cyclic stretch. The statistics of cell morphological axis before and after 3 h of cyclic stretch was shown in Figure 6(b). Notably, most cells realigned to 60°–70° with respect to external stretch direction, instead of

FIG. 6. Realignment of cell morphology and actin cytoskeleton under cyclic directional stretch. (a) Time-lapse 10 $\times$ phase contrast images of REF-52 cells undergoing morphological remodeling during cyclic directional stretch (white arrows). (Insets) Zoom-in images showing the morphological realignment of a single cell. Scale bar: 200 μ m. (b) Statistics of the orientation of cell body axis before and after 3 h cyclic stretch, demonstrating cells preferentially realigned at 60°–70° with respect to the stretch direction ($n_{\text{cell}} = 100$ for each time point). (c) Immunofluorescence images of actin stress fibers after 3 h cyclic stretch (white arrow), as a demonstration of the polarization of intracellular structures. Scale bar: 20 μ m.

realigning completely perpendicular (90°) to it. This observation is consistent with those from others.¹⁵

V. DISCUSSION

Our cell stretching device provided a convenient platform for studying the fundamental mechanosensitive behaviors of biological systems from multicellular to subcellular scales. Besides FAs, our device could potentially be applied to study force-dependent dynamics of other subcellular machineries such as clathrin-coated pit and caveolae. Using voltage-sensitive dyes, this device could also be applied to study the behavior of mechanosensitive ion channels as well as intracellular calcium signaling in response to stretch, which has been shown important for regulating mechanoresponsive contractile behaviors of vascular smooth muscle cells.¹⁰

Complex integrated systems could also be built upon the platform established by our device, in order to mimic a multi-parametric dynamic cell microenvironment and thus to study mechanobiology questions where multiple mechanical cues can affect cellular behaviors and functions in a synergistic or

competitive fashion. For example, by conjugating the PDMS membrane with polyacrylamide gels of different mechanical stiffness, our device could be applied to study how substrate stiffness and mechanical stretch co-regulate cell morphology and other cellular phenotypes. In addition, by replacing the PDMS cell culture well with a PDMS microfluidic chamber, our device could possibly be adapted to study emergent cellular behaviors under regulations by both shear stress and tensile stretch, which serves as a better *in vitro* mimic of *in vivo* cardiovascular microenvironment.

VI. CONCLUSION

In this study, we have developed a device for dynamic directional cell stretching and real-time subcellular imaging *in situ*. Using a cam and follower mechanism and applying a standard mathematical model, it was possible to regulate the dynamic stretch waveform via rationally designing the geometry and dimension of the cam. We demonstrated the utility of our device in two applications: (1) *in situ* imaging of force-mediated FA dynamics in response to step-function stretch and (2) the realignment of cells and the F-actin cytoskeleton in response to long-term cyclic directional stretch. In all, this work was the first development of a dynamic cell-stretching device using the cam-and-follower mechanism, which is straightforward for stretch modification and does not involve complex pneumatic systems (like in the most advanced Flexcell system). Our device could have broad potential applications in studying cell mechanotransduction and mechanobiology where external mechanical forces play an important role in regulating behaviors of subcellular molecular systems or multicellular constructs.

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