Journal of Visualized Experiments

Live cell imaging during mechanical stretch

--Manuscript Draft--

Manuscript Number:	JoVE52737R3
Full Title:	Live cell imaging during mechanical stretch
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Mechanotransduction; bioreactor; mechanobiology; over distention; pulmonary epithelium; acute lung injury
Manuscript Classifications:	7.1.154.100.440: Mechanotransduction, Cellular; 7.1.595.100.440: Mechanotransduction, Cellular; 7.4.299.880.580: Mechanotransduction, Cellular; 93.37: Mechanical Engineering; 95.51.8: bioengineering (general)
Corresponding Author:	Esra Roan, Ph.D. University of Memphis Memphis, TN UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	eroan@memphis.edu
Corresponding Author's Institution:	University of Memphis
Corresponding Author's Secondary Institution:	
First Author:	Gabriel Rapalo
First Author Secondary Information:	
Other Authors:	Gabriel Rapalo
	Josh D Herwig
	Kristina R Wilhelm
	Robert Hewitt
	Christopher M Waters
Order of Authors Secondary Information:	
Abstract:	There is currently a significant interest in understanding how cells and tissues respond to mechanical stimuli, but current approaches are limited in their capability for measuring responses in real time in live cells or viable tissue. A protocol was developed with the use of a cell actuator to distend live cells grown on or tissues attached to an elastic substrate while imaging with confocal and atomic force microscopy (AFM). Preliminary studies show that tonic stretching of human bronchial epithelial cells caused a significant increase in the production of mitochondrial superoxide. Moreover, using this protocol, alveolar epithelial cells were stretched and imaged, which showed direct damage to the epithelial cells by overdistention simulating one form of lung injury in vitro. A protocol to conduct AFM nano-indentation on stretched cells is also provided.
Author Comments:	Dear Editors of the Journal of Visualized Experiments Journal: We are submitting a revised version of our manuscript titled "Live cell imaging during mechanical stretch" for your consideration of publication as a protocol in the Journal of Visualized Experiments. Our manuscript presents a novel method that we developed in our lab along with a new mechanical device for stretching of cells and tissue in culture. We have revised the manuscript as per the Editors' and reviewers' suggestions. The work presented in this manuscript has not been submitted to any other journal. All

	the authors listed have contributed significantly to this manuscript and have approved this current version. We look forward to hear back from JoVE. Sincerely; Esra Roan, PhD
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Title:
Live cell imaging during mechanical stretch

Authors:
Gabriel Rápalo
Department of Physiology/Biomedical Engineering and Imaging
UTHSC
Memphis, TN, 38163
grapalo@uthsc.edu, gabrielrapalo@gmail.com

Josh D. Herwig
Department of Biomedical Engineering
University of Memphis
Memphis, TN, 38152
JDHerwig@gmail.com

Robert Hewitt
Department of Engineering Technology
University of Memphis
Memphis, TN, 38152
robert.w.hewitt@gmail.com

Kristina R. Wilhelm
Department of Physiology/Biomedical Engineering
UTHSC
Memphis, TN, 38163
kwilhel1@uthsc.edu

Christopher M. Waters
Department of Physiology/Biomedical Engineering
UTHSC
Memphis, TN, 38163
cwaters2@uthsc.edu

Esra Roan
Department of Biomedical Engineering
University of Memphis
Memphis, TN, 38152
eroan@memphis.edu

Corresponding author:
Esra Roan, Ph.D.
eroan@memphis.edu

Keywords:
Mechanotransduction, bioreactor, mechanobiology, overdistention, pulmonary epithelium, acute lung injury

Short Abstract:
A novel imaging protocol was developed using a custom motor-driven mechanical actuator to allow the measurement of real time responses to mechanical strain in live cells. Relevant to mechanobiology, the system can apply strains up to 20% while allowing near real-time imaging with confocal or atomic force microscopy.

Long Abstract:
There is currently a significant interest in understanding how cells and tissues respond to mechanical stimuli, but current approaches are limited in their capability for measuring responses in real time in live cells or viable tissue. A protocol was developed with the use of a cell actuator to distend live cells grown on or tissues attached to an elastic substrate while imaging with confocal and atomic force microscopy (AFM). Preliminary studies show that tonic stretching of human bronchial epithelial cells caused a significant increase in the production of mitochondrial superoxide. Moreover, using this protocol, alveolar epithelial cells were stretched and imaged, which showed direct damage to the epithelial cells by overdistention simulating one form of lung injury *in vitro*. A protocol to conduct AFM nano-indentation on stretched cells is also provided.

Introduction:
Cells are subjected to mechanical loads in many tissues, and this mechanical stimulation has been shown to promote changes in patterns of gene expression, release of growth factors, cytokines, or remodeling of the extracellular matrix and cytoskeleton[1-4]. The intracellular signals transduced from such mechanical stimuli occur through the process of mechanotransduction[5-7]. In the respiratory system, one outcome of mechanotransduction is the increase in reactive oxygen species (ROS)[8,9] and pro-inflammatory cytokines[10] in pulmonary epithelial cells in the presence of cyclic tensile strain. Strong evidence also suggests that excessive tensile strain leads to direct injury to the alveolar epithelium, in addition to the biochemical responses of cells[11-14]. Although the focus here is primarily on the response of lung cells to mechanical deformation, pathways induced by mechanotransduction play a key role in the basic function of many tissues in the human body, including the regulation of vascular tone[15] and the development of the growth plate[16].

The growing interest in mechanotransduction has resulted in the development of numerous devices for the application of physiologically relevant mechanical loads to cultured cells and tissue. In particular, devices applying a tensile strain, which is a common form of mechanical loading experienced by tissue, are popular [11,17-19]. However, many of the available devices are either designed as a bioreactor for tissue engineering applications or are not conducive to real time imaging with stretch. As such, there is a need to develop tools and methods that can visualize cells and tissues in tension to facilitate the investigation of pathways of mechanotransduction.

Herein, an in-plane mechanical stretching device was designed and protocols were developed to apply multiple forms of strain to tissues and cells while allowing imaging of the biochemical and mechanical responses in real time (**Figure 1A-D)**. The device utilizes six evenly spaced clamps arranged circumferentially to grasp a flexible membrane and apply an in-plane, radial distention up to approximately 20% (Figure 1B). The actuating device can be placed in a cell culture incubator for an extended period of time, while the motor (Figure 1C) is positioned outside the incubator and controlled by proprietary software provided by the motor supplier. The motor is connected to a linear driver, which rotates an internal cam, driving the six stretcher clamps uniformly in tension and relaxation.

In addition to the mechanical device, customized flexible membranes were created from commercially available cell culture ready membranes to be used in the mechanical system. Then circular walls (with a diameter of approximately 28 mm) were made and attached on to the flexible membrane so that cells could be cultured only in this region of well-described strain profile. In order to determine whether the placement of these membranes within the actuating device would provide uniform and isotropic strain in the center of the flexible membrane, finite element analysis was conducted using commercially available software (Figure 1E-F). The flexible membrane was modeled with symmetric boundary conditions and utilizing all quadrilateral elements for the mesh. The concentric rings seen in the contour plot of maximum principal strain shown in Figure 1F indicate the isotropic distribution of the strain.
The strain experienced by the membrane was measured by recording images of markings through loading (Figure 2). Figure 2D shows that the average membrane strain measured in radial and axial directions was approximately linear with respect to the applied motor counts up to a maximum linear strain of 20%. There was no significant difference between the strain levels measured during distention compared with those measured during retraction back to the resting position. Next, the displacement of human bronchial epithelial cells (16HBE) and their nuclei cultured on the custom flexible membrane were measured. Fluorescently labeled (DAPI) nuclei of the 16HBE cells were imaged using a 20X objective under a confocal microscope, whereas whole cell displacement was measured with phase contrast images recorded with a digital microscope. As seen in **Figure 3**, the strain measured by displacement of nuclei was similar to that measured by displacement of markings on the membrane, up to ~20% linear strain. This confirms that the strain applied to the membranes was transmitted to the adherent cells. The protocols describing the use of the custom device on a traditional microscope and an atomic force microscope are provided in the following steps.

Protocol:

1. Construction of membrane with well walls for retention of cell culture media (see Figure 1D for the final product).

1.1. Using polydimethylsiloxane (PDMS) sheets coated with Collagen I, cut the outline of the flexible membrane with a scalpel or a die.

1.2. Place each membrane in a 60 mm petri dish for storage.

1.3. Creation of walls:

1.3.1. Mix PDMS at a 10:1 weight ratio of elastomer A to elastomer B (curing agent).

1.3.2. Pour 5 mL of fully mixed PDMS into 50 mL tubes.

1.3.3. Place 50 mL tubes with uncured PDMS horizontally in a hybridization oven.

1.3.4. Use the rotor function to coat the inner walls of the tubes at 8 rpm during the curing time. At room temperature, the PDMS will be fully cured in 2 days.

1.3.5. Remove the PDMS from the tube in a sterile cell culture hood.

1.3.6. Using a new razor blade, partition the cylinder of PDMS into sections 4 mm in height.

1.3.7. Place the sections, which will serve as the membrane walls, into a petri dish container without allowing the walls to become creased.

1.4. Select and center one wall of PDMS on one membrane while maintaining the two in the petri dish.

1.5. Gently place uncured PDMS (10:1 ratio) on the outside perimeter of the wall to be used as glue. Prevent formation of gaps between the wall and the membrane since it is there to retain liquid.

1.6. Place each petri dish containing the completed membrane and covered in an oven at 70 °C for 24 hours to cure.

2. Correlation of motor rotations with clamp displacement or radial growth for calibration (Figure 2).

2.1. Start the software controlling the motor.

2.2. Displace the clamps of the device using the manual setting in the motor software.

2.3. Measure the distance and record the motor count position between opposing clamps at both minimum and maximum displacement of the clamps.

2.4. Calculate the percent change in the distance between the clamps as a function of the motor count (~0-75k counts). This will indicate the maximum potential strain in achieved on the membrane.

3. Application of stretch on mouse lung epithelial cell line (MLE12).

3.1. Seed MLE12 cells at 2.5 million cells on the flexible membrane (Step 1) per well to be confluent within two days. The seeding density may vary for different cell types.

3.2. Make sure the mechanical actuator is in a fully relaxed position.

3.2.1. Remove cell culture media.

3.2.2. Using 1.5 mm biopsy punches, punch two holes in each of the six clamp (See Figure 1D) tabs of the membrane. The radial placement of the holes determines the amount of pre-tension the membrane experiences initially. Punch the holes at a radius of 20.5 mm on the membrane tabs if no pre-tension is desired.

3.2.3. Position the membrane on the stretcher with the punched holes lining up with the pins within the clamps. Place top clamps in place. Tighten the screws one at a time alternating sides.

3.2.4. Add 1 mL cell culture media.

3.3. Place the device on the microscope stage centering the middle of the membrane with the light path.

3.4. Use tape or magnets (if possible) to fix the device to the stage. Once fixed, control the in-plane (parallel to the membrane) and vertical (z-direction) of the mechanical device with the stage controller of the microscope.

3.5. Apply stretch with the software provided by the manufacturer to the motor or manually controlling the position and speed of motor rotation[20].

4. Measurement of mitochondrial reactive oxygen species (ROS).

4.1. Once cells are confluent, add 1-2 mL of room temperature DMEM with mitochondrial superoxide indicator (5 µM final concentration) directly on the cells.

4.2. Incubate for 10 minutes at 37 °C.

4.3. Wash cells gently three times with buffer warmed in a water bath to 37 °C.

4.4. Immediately place the flexible membrane on the stretcher (Step 2.2) already in place under an upright confocal microscope.

4.5. Add 1 mL of DMEM with phenol-red free medium and 25 mM of HEPES.

4.6. Set excitation/emission filters to 510/580 nm.

4.7. Image multiple fields every 15 minutes to create the desired time course of mitochondrial superoxide production.

4.8. From captured images, record fluorescence intensity histograms at each time interval using software capable of quantifying fluorescence intensity.

5. Application of stretch and atomic force microscopy (AFM).

NOTE: These steps are provided for a specific AFM and optical microscope combination. (Figure 4 and Materials List).

5.1. Prepare the AFM for the experiment.

5.1.1. Increase the height of the AFM head to its maximum position in the z-direction.

5.1.2. Put extenders on the legs of the AFM to lift the plane at which AFM cantilever contacts the sample. The specimen and the AFM head needs to be lifted to accommodate the height of the mechanical device.

5.2. Prepare the optical microscope (if available).

5.2.1. Remove the AFM scanner plate. Remove the desired objective.

5.2.2. Add a spacer to the objective. The height of the spacer would depend on the objective and the specific AFM set-up, but it is necessary if optical imaging is desired since observation plane will be shift in the z-direction by an amount equal to the stretcher and adapter height (Figure 5A). Note that the AFM usually provides low magnification imaging from an optical path above the device.

5.2.3. Mount the desired objective back in its location. Place the scanner back on to the AFM.

5.2.4. Start the AFM software. Start all necessary light sources including the light source for fluorescence measurements. Focus the optical microscope on the cells and start its software.

5.2.5. Mount a chip with a cantilever beam that is appropriate for the desired measurements. 200 pN/nm or less stiffness is preferred when measuring the elastic modulus of live cells.

5.2.6. Align the laser and calibrate the cantilever stiffness according to manufacturer's suggestions on a glass coverslip mounted on the device.

5.3. Prepare a membrane as in Step 1, but with the following modifications.

5.3.1. Immediately before mounting the membrane on to the stretching device, cut the walls to about 1 mm in height. This prevents the interference experienced between the membrane walls and the load cell.

5.3.2. Mount the membrane on the mechanical device as described 3.2.1-3.2.3.

5.3.3. Remove cell culture media to prevent damage to the AFM scanner in case of a spill.

5.3.4. Couple the device with an adapter (Figure 5A). Place the mechanical device with the adapter on the scanner.

5.3.5. Stretch the membrane to the desired tensile strain level.

5.4. Nano-indentation of stretched cells.

5.4.1. Add a limited (<0.5 mL) volume of media on the cells to avoid AFM scanner or microscope damage due to a spill.

5.4.2. Engage the cantilever beam with the membrane.

5.4.3. Follow the protocol of the particular AFM device to scan areas of interest.

Representative Results:

Reactive Oxygen Species and Deformation

Previous studies have shown increases in reactive oxygen species (ROS) in airway and alveolar epithelial cells in response to cyclic stretch[21]. Reactive oxygen species include molecules and free radicals derived from molecular oxygen with high reactivity to lipids, proteins, polysaccharides, and nucleic acids[22-24]. ROS serve as a common intracellular signal to regulate ion channel function, protein kinase/phosphatase activation, and gene expression, but excessive or unregulated production can contribute to apoptotic and necrotic cell death, neurodegeneration, atherosclerosis, diabetes, and cancer[24,25]. Superoxide, a pre-cursor for other forms of ROS, can be produced as a by-product of mitochondrial respiration when electrons leak from the electron transfer chain. Previous studies suggested that cyclic mechanical stretch stimulated the production of superoxide through a combination of the NADPH oxidase system (nicotinamide adenine dinucleotide phosphate-oxidase) and the mitochondrial electron transport chain (complex I and III). It has been proposed that this stimulation was due to a direct distortion of the mitochondria[21] possibly due to connections to the cell cytoskeleton. In order to test this hypothesis, the cell stretching device was developed so that we could image changes in ROS production in live cells in response to mechanical stretch. Here, the mitochondrial ROS produced by bronchial epithelial cells due to stretch was measured using the custom device and protocol. In the absence of mechanical stretch, ROS production did not significantly increase over 60 min (Figure 4). When a single 17% stretch was applied and maintained, there was an increase in mitochondrial ROS that persisted for another 60 min.

Direct Epithelial Monolayer Damage Due to Stretch

Indirect mechanisms by which excessive stretch in the lung epithelium harms the lungs has been discussed in depth elsewhere[2]. Several studies suggest that excessive mechanical stretch of lung epithelial cells can cause direct injury involving damage to cells[11,13,26,27]. This type of damage is difficult to capture without real-time imaging during the mechanical distention. As such, utilizing the custom device and phase contrast microscopy consecutive images of cells that were mechanically stretched were recorded before and after treatment with a pro-inflammatory cytokine (50 ng/mL TNF-α for 6 hours) (Figure 5). Images of the same field of cells were captured at increasing strain levels. The images show the formation of a gap when the cells were stretched between 10 and 15% strain as indicated by the red arrows. It is important to note that these images were recorded in near real-time, <10 seconds of lag between the end of stretch and imaging, while the cells were stretched. In previous studies of cell imaging of stretched cells, images were obtained before and after stretch comparing only the images of cells not at stretched condition[11,13,28].

AFM Nano-indentation on stretched cells

The mechanical device was placed in the AFM with the use of an adapter plate (see Figure 6A). Using previously published methods[13], elastic modulus maps (E-maps) of MLE12 cells before and after 10% tensile strain were obtained (Figure 6D-E). Although phase contrast image at 10% strain (Figure 6D) was obtained within 10 seconds of the stretch, the E-maps shown in Figure 6(D-E) consumed approximately 22 minutes with 300 individual force-deflection curves recorded over a 40 μm X 40 μm area with a 2.5 μm/s velocity of the tip in the z-direction.

Figure 1: The custom designed device for cell culture and stretching. (*A*) Computer-generated drawing of the mechanical design of the device, (*B*) photograph of the completed device, *(C)* photograph of device connected to the motor and linear driver, which converts the motor rotation into a 1-dimensional motion which controls the clamp biaxial stretch of the *(D)* silicone rubber membrane. The substrate contains two holes on each clamp tab to be placed on posts on each clamp. (*E)* Dimensioned FEA model of the flexible membrane that is $^1/_4$ of the whole structure due to use of symmetric boundary conditions. *(F)* Maximum principal strain is shown in the full membrane depicting the formation of rings indicative of isotropic strain field.

Figure 2: (A) The device supporting a flexible membrane with markings applied to measure applied strain is shown. Close-up images of the markings, before (B) and after (C) stretch, were labeled with an arrow to clearly indicate the displacement of the markings on the membrane. (D) Membrane strain as a function of the motor counts during approach and retraction. Membrane strain was similar in both approach and retraction with retraction producing slightly higher strains.

Figure 3: Membrane strain transmission to the cells. (A-B) Phase contrast images of human bronchial epithelial cells (16HBE) on the flexible membrane using a 10X objective, before (A) and after (B) 20% strain. (C-D) Fluorescently labeled (DAPI) nuclei of the 16HBE cells were

imaged using a 20X objective under a confocal microscope, before (C) and after (D) 20% strain. (E) The membrane strain experienced by cells was linear and homogenous.

Figure 4: The impact of a single stretch on the ROS production of 16HBE cells was observed using a cumulative mitochondrial superoxide sensor using a 20X objective under a confocal microscope. (A-C) Snapshots of the time course (0, 60 min, and ~65 min) of superoxide production during stretch in the same field of cells. (D) Relative fluorescence intensity of 16HBE cells over time indicating a 50% increase in fluorescence intensity from baseline production of superoxide within the first hour (A to B).

Figure 5: Phase contrast images of an alveolar epithelial (MLE12) monolayer response to increasing stretch. (A-D) were all recorded using a digital microscope with a 20X objective. The arrows indicate the locations where cell-to-cell separation occurred.

Figure 6: A) Mechanical device shown in the AFM for nano-indentation of cells with stretch. The scanner must be removed for the placement of objective extender. Three leg extenders are attached to all three legs of the AFM head to allow for device placement. An adapter plate is utilized to mount the stretcher on to the AFM scanner (black plate). The cantilever beam scans in the direction of the arrow shown in the before stretch phase contrast image an MLE12 cell (B). The red squares in the before (B) and after (C) stretch images depict the area that was captured during nano-indentation (40X objective). The elastic modulus maps, before (D) and after (E) stretch, are obtained with previously published analysis techniques[13].

Discussion:

A unique device for live cell imaging during mechanical stretch was developed; and this device was used in a protocol to study lung epithelial cell mechanobiology. In preliminary studies, it was found that a single held stretch stimulated the production of mitochondrial superoxide in bronchial epithelial cells. In addition, it was demonstrated that increased levels of mechanical strain caused direct damage to the integrity of a monolayer of alveolar epithelial cells.

To conduct these preliminary experiments, the device was first calibrated, and then it was shown that the membrane strain was transmitted to cells (Figures 2-3). Overall the device performed well, as indicated by the close correlation between the membrane strain (measured by marks on the membrane) and the cell strain (measured by distances between nuclei) shown in Figure 3E. However, there were some minor differences observed between strain measured on the membranes during the approach and the return curves of the markers, as shown in Figure 2D. This was likely due to backlash in the device and possibly related to gripping issues. In addition, a universal mounting mechanism for the device is needed so that it can efficiently be used on microscopes with different configurations. This would reduce the time spent between positioning of the device and image acquisition while maintaining a robust connection to the microscope. Holes punched in the membranes were used to apply a minor pre-strain to the membrane to prevent formation of an initial compressive state due to clamping of an elastic material. The compressive state also leads to a reduction of maximum applicable strain. The custom device was designed to fit in an Asylum MFP3D Atomic Force Microscope to allow nano-indentation of live cells during mechanical stretch. We are not aware of any other device that is capable of inducing an isotropic stress state in the imaging plane that can function within an AFM. The device was also designed for continuous use in a cell-culture incubator (kept at 37 °C in a humid, sterile environment with 5% CO_2). The ability to function within an incubator allows the study of cells undergoing long-term stretch regimens.

There are several limitations to the presented approach. First, as the membrane stretches, the field of focus moves out of plane, although minimally (~100 µm). This becomes a limitation when tracking a field of cells through a stretch regimen, because the field of view changes over the course of the membrane distention. Even though membrane within the 22 mm diameter experiences 0.23-0.25 strain, the regions outside of this inner diameter experience a more variable strain field. Although imaging studies are easily limited to this central region, it is possible that the cells responding to higher levels of strain at outer regions can impact the response of cells within the central region. A uniform strain distribution over a larger area of interest can be achieved with improvements in the membrane design. The PDMS membrane is auto-fluorescent at wavelengths commonly used for fluorescent indicators, and this limits the capability for imaging fluorescence in cells from beneath the membrane. For this reason an upright microscope was utilized with water immersion objectives in our studies of ROS production described above. This can be a limitation for studies in which AFM and fluorescence imaging are combined.

The most common commercial device (FlexCell FX 5000 Tension System) for applying mechanical strain to cells incorporates a controllable vacuum system applying tensile strain on a membrane while moving in and out of the optical plane. The membrane and clamp design

presented herein create a bi-axial strain field where strain is isotropic and remains in plane allowing real-time imaging. With minor modifications to the membrane design, the stretcher can produce uniaxial stretch modes as well. If the cells are first seeded on a distended membrane, in-plane compressive loads can also be applied. The device is able to reach both physiological and pathological levels of strain. In summary, a new device and protocols were developed that can be used to apply mechanical strain to live cells that can be imaged using fluorescence microscopy and AFM nano-indentation.

Disclosures:
The authors declare that they have no competing financial interests.

Acknowledgments:
The authors would like that thank Fedex Institute of Technology at the University of Memphis for their support. The authors would like to acknowledge students of the senior design project group in the Mechanical Engineering Department at the University of Memphis (David Butler, Jackie Carter, Dominick Cleveland, Jacob Shaffer), Daniel Kohn from the University of Memphis Engineering Technology department for motor control, and Dr. Bin Teng and Ms. Charlean Luellen for their help in cell culture. This work was supported by K01 HL120912 (ER) and R01 HL123540 (CMW).

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Figure 1
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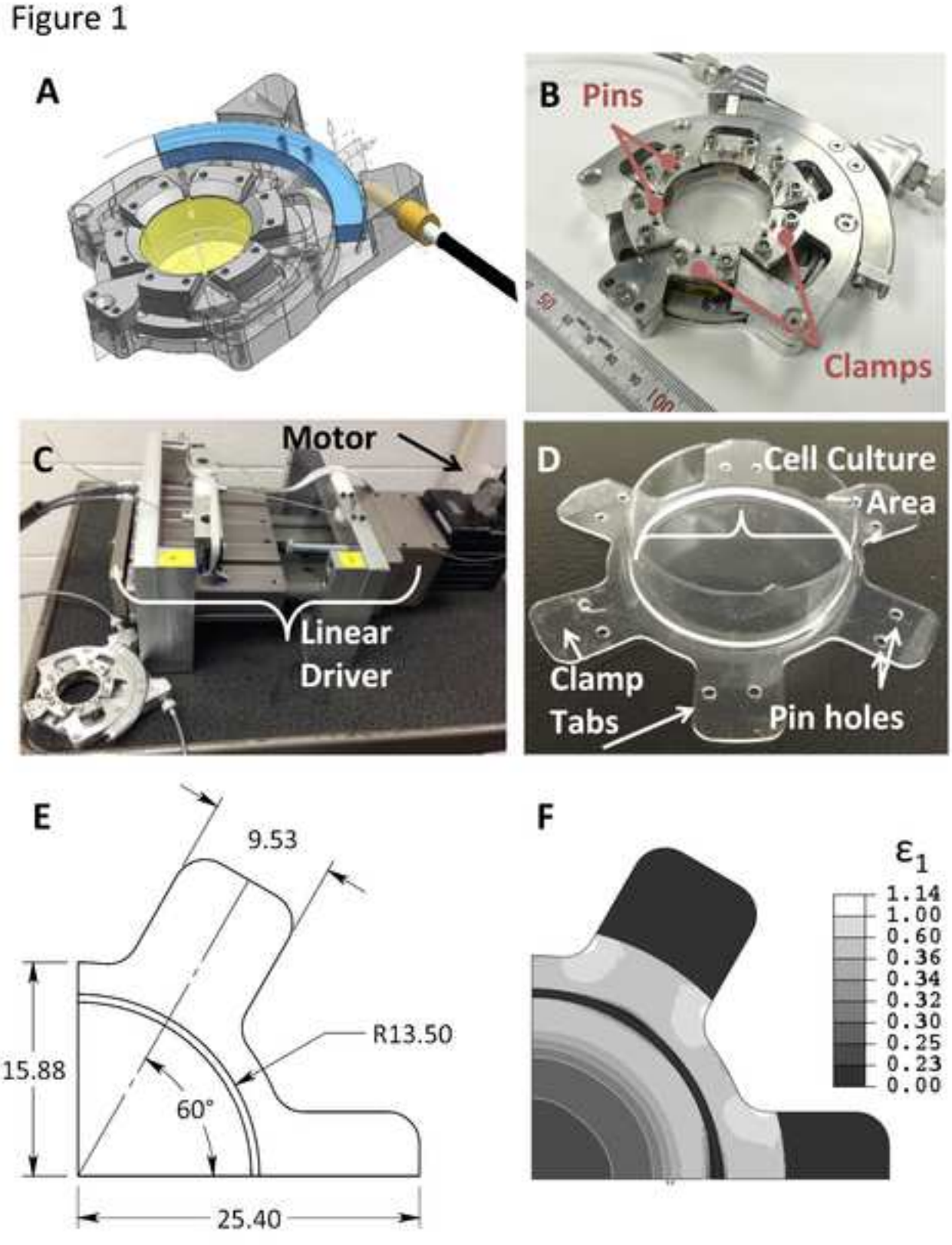

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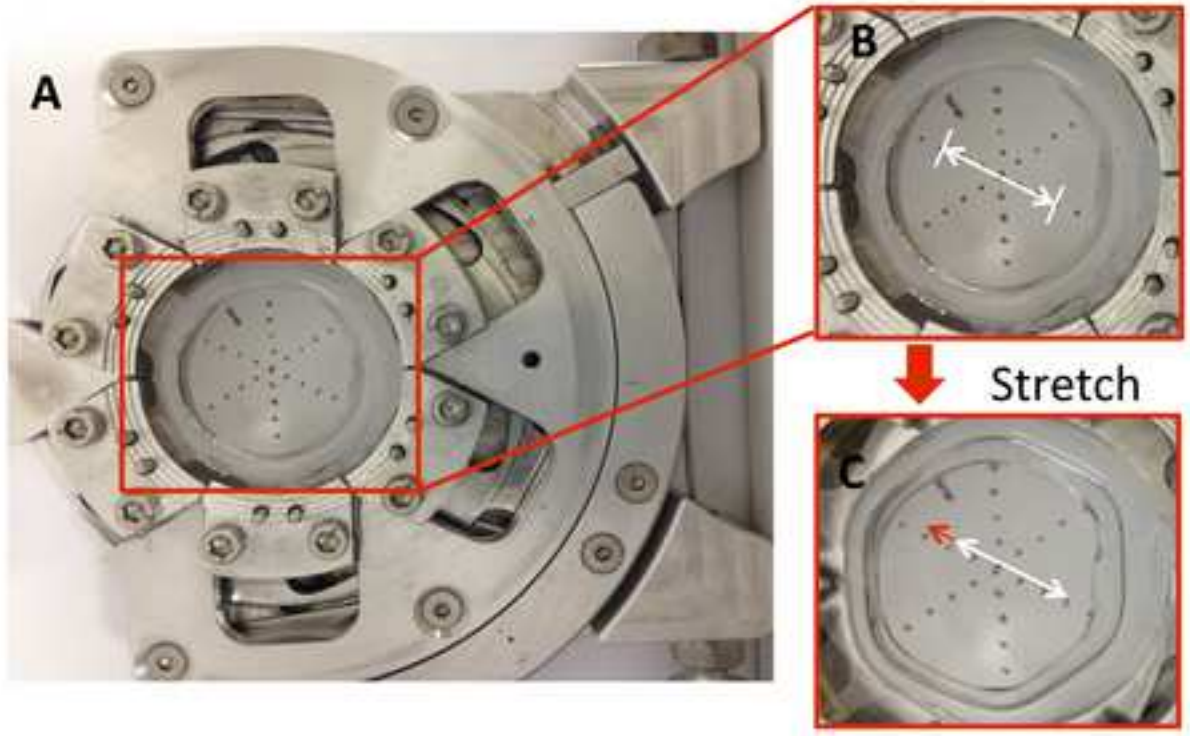

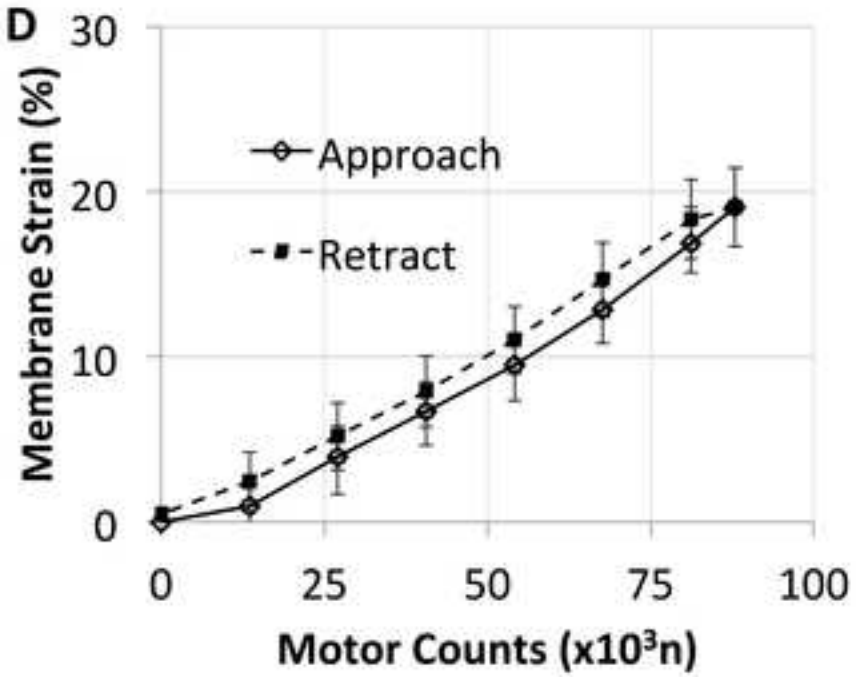

Figure 3
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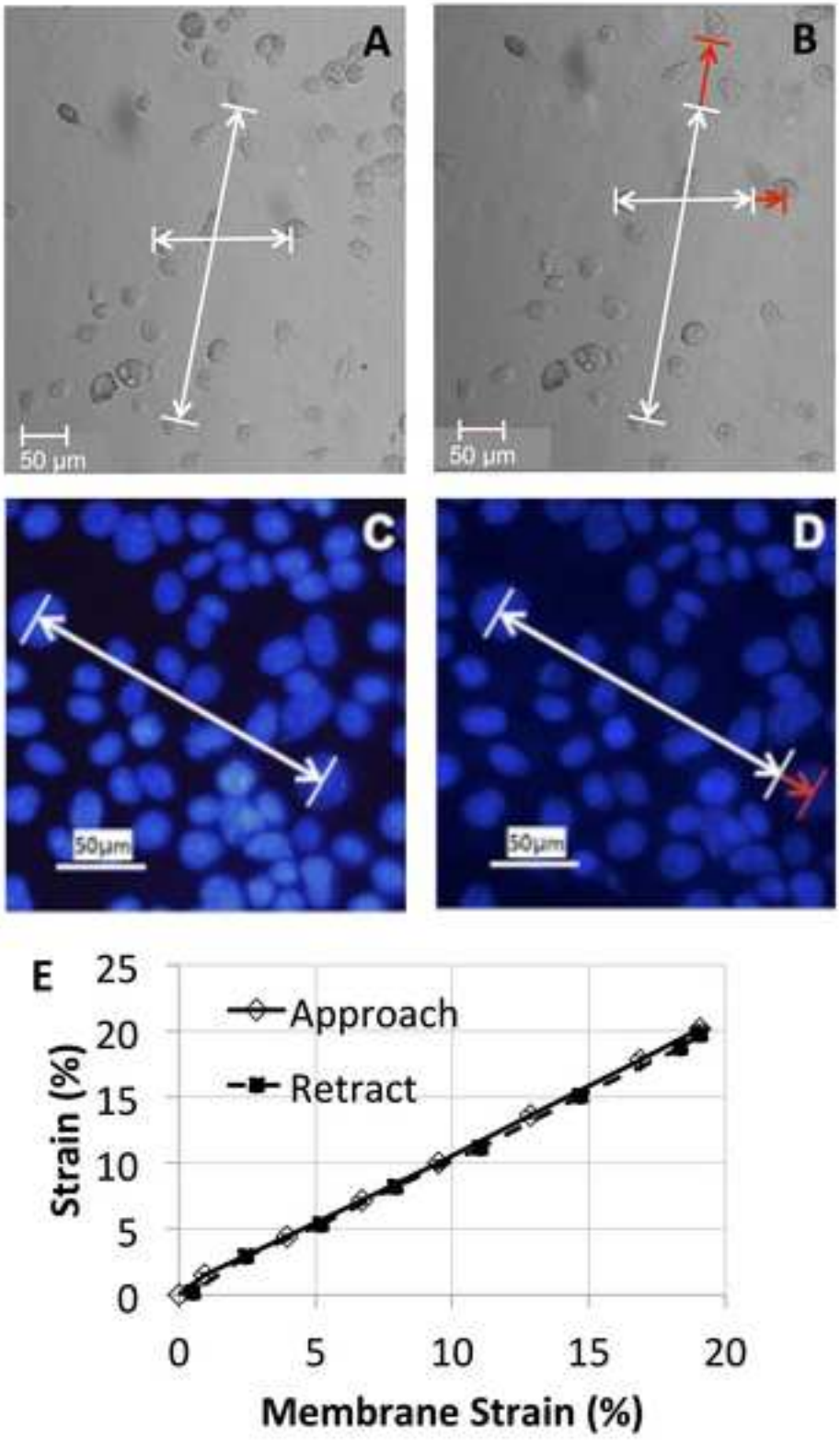

Figure 4
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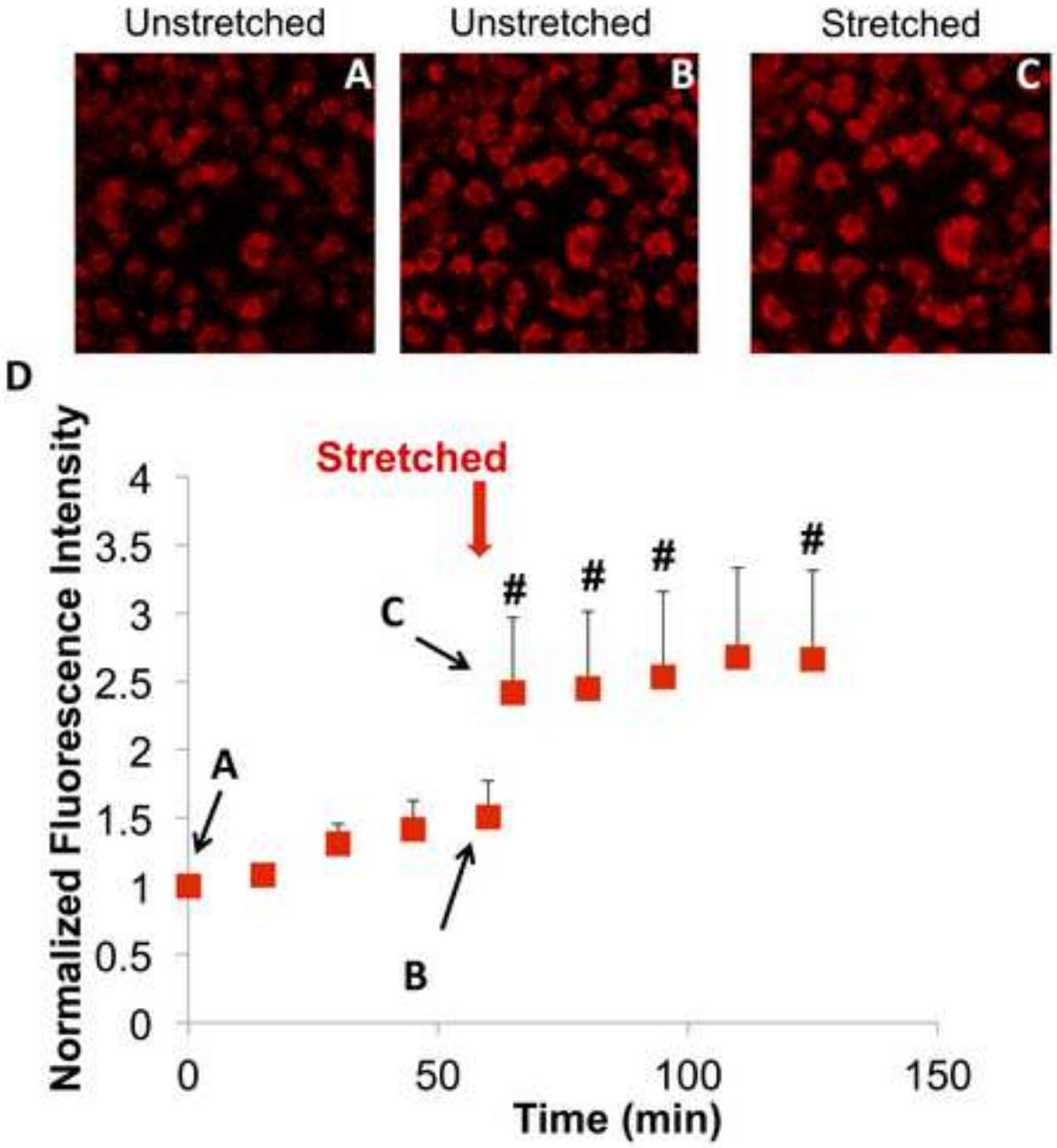

Figure 5
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Figure 5

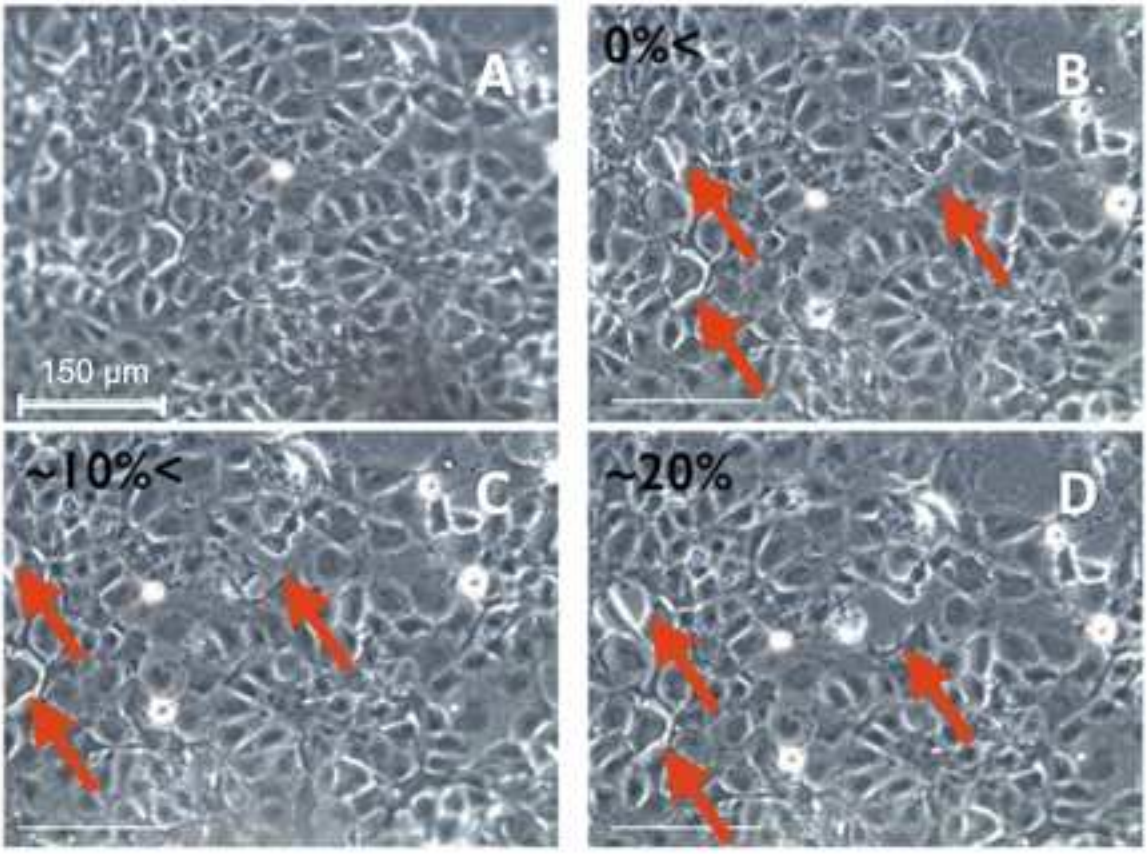

Figure 6
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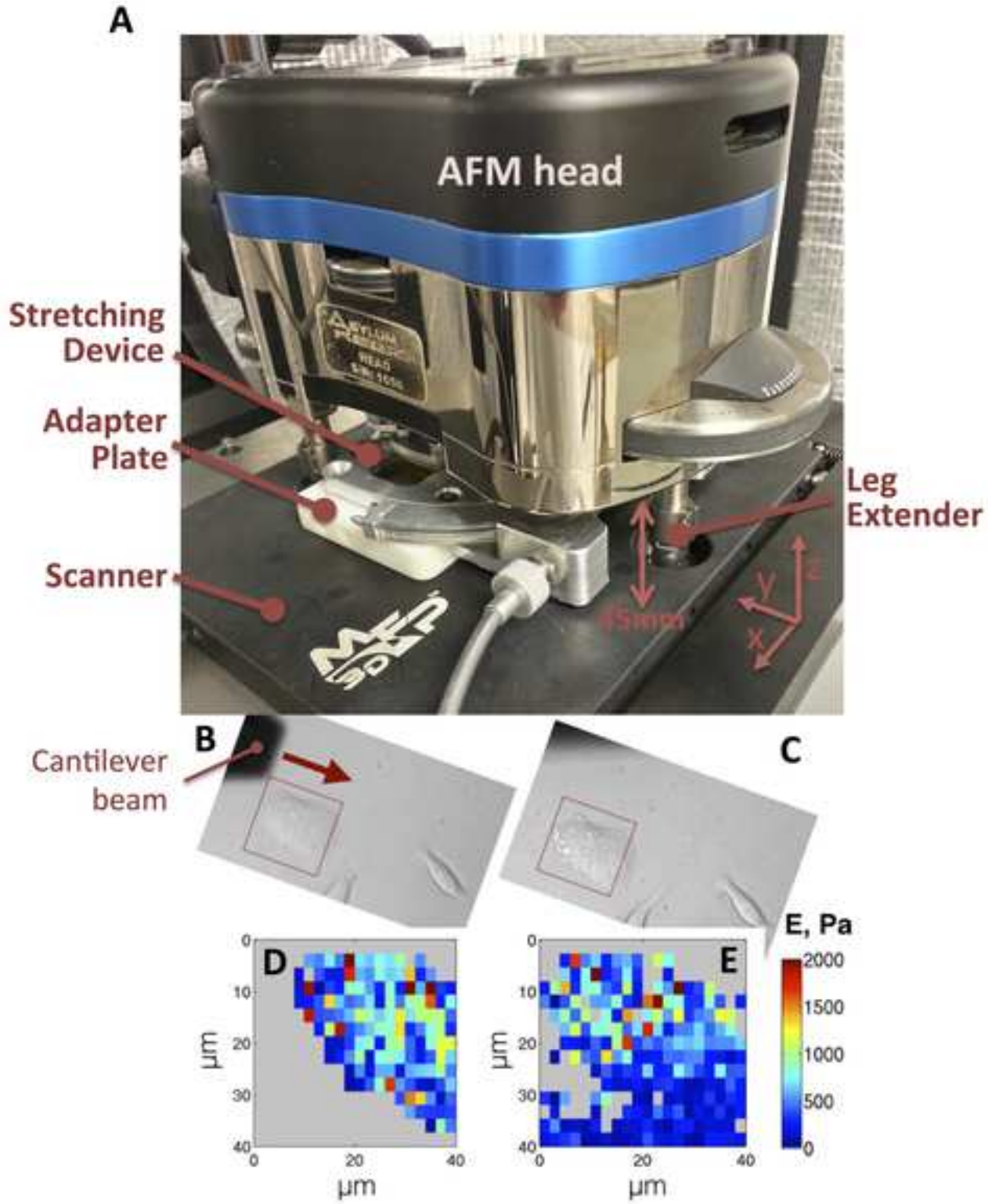
Figure 6



Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
SmartMotor NEMA 34: 3400 Series	MOOG Animatics	SM3416D
Flexcell Membrane (Collagen I coated)	Flexcell International Corp	SM2-1010C
Sylgard 184	Dow Corning Corporation	
Hoechst 33342	Sigma-Aldrich	H1399
MitoSOX	Sigma-Aldrich	M36008
Tiron	Sigma-Aldrich	D7389
DMEM		
FBS		
HEPES		
50 ml tubes	Fisher Scientific	06-443-19
Hybridization oven	Bellco Glass	
MLE12 Cells	ATCC	CRL-2110
16HBE cells	ATCC	CRL-2741
AFM Indentation Experiments		
Cantilever Beams for Nano-indentation	Budget Sensors	Si-Ni30
AFM	Asylum Research	MFP3D
Olympus microscope	Olympus	IX-71
AFM Leg Extenders	Asylum Research	Not available
Finite Element Analyses		
ABAQUS	Simulia	6.12
Software		
ImageJ	NIH	
Microscopes		
Digital microscope	Life Technologies	EVOS XL Core

Confocal microscope	Zeiss	LSM 710

Comments/Description	Location
Integrated motor, controller, amplifier, encoder and communications bus	Santa Clara, CA
3.5x5.25x0.020"	Hillsborough, NC
10:1	Midland, MI
DAPI stain	St. Louis, MO
	St. Louis, MO
mitochondrial superoxide label	St. Louis, MO
superoxide inhibitor	
Any centriguge tube can be used to create an area for imaging.	
	Vineland, NJ
Mouse Lung Epithelial Cells	Manassas, VA
Human Bronchial Epithelial Cells	Manassas, VA
	Sofia, Bulgaria
	Santa Barbara, CA
Inverted microscope with 20X and 40X objectives.	
AFM microscope	Santa Barbara, CA
	Providence, RI
Initially a self standing company, now owned by Life Technologies.	Carlsbad, CA

2-photon upright microscope	Jena, Germany

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Author(s): Gabriel Rapalo, Robert Hewitt, Kristina Wilhelm, Chris Waters, Esra Roan

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CORRESPONDING AUTHOR:

Name:	Esra Roan
Department:	Biomedical Engineering
Institution:	University of Memphis
Article Title:	Live Cell imaging during mechanical stretch
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We thank the reviewers and the editor for their interest in improving our manuscript. We addressed a number of common criticisms in the "Responses to All Reviewers" section and then addressed unique comments within each Reviewer's section.

Responses to the Editor:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We have edited the protocol extensively and we feel confident that the new version is much improved.

2. What surface are the cells seeded on in step 3.1?
The cells are seeded on PDMS membranes coated with Collagen 1 purchased from (FlexCell Int.). We added this description in the protocol.

3. Please clarify step 4.1.
A final concentration of 5uM mitochondrial superoxide indicator was achieved in 1-2mL volumes of DMEM. We refrained from including a volume since stock solutions may be prepared in various concentrations.

4. Length Warning: The length is currently at our upper limit, so if material is added following peer review it may become necessary to cut something from the material to be filmed.
We would like to discuss this further as we are concerned with not having room to include all of the details suggested by the reviewers. We added material based on comments and additional experimental results were added. We also would like to note that there are two imaging modalities with a high level of customization potential. As such, providing all of the details may not help the users as much.

We want to also note that we have highlighted different parts of the protocol for filming.

Responses to All Reviewers

1. Strain distribution:

We designed membrane to produce a near uniform and isotropic strain field over the region where we focus our imaging and AFM studies. We initially utilized finite element analysis and then actual measurements to show this. We agree with Reviewer 5 that we cannot expect a perfect match between FEA predictions and actual device due to manufacturing tolerances. However, in Fig. 2D, we show the average change in length between markings as a function of motor counts collected from the radial as well as axial direction, which exhibit a low level of variation as seen in the error bars.

In light of these comments, we updated Fig. 1E to reflect the variation in the maximum principal strain. For example, the centermost gray area, falling within 22 mm diameter, experiences a strain variation falling between 0.23-0.25. It is important to note that this is a high level of strain for cells and that as the applied strain tends to a lower value, the variation will also decrease.

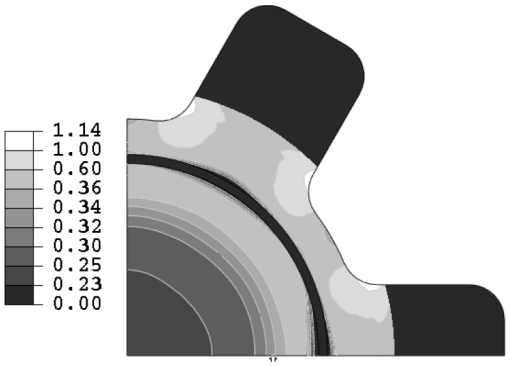

Fig. 1: Maximum principal strain distribution. Dark gray areas belong to the clamped regions. There are stress concentrations near the clamps that do not reach the center of the membrane. Quarter of the membrane is considered for finite element modeling, quad elements are utilized predominantly, and displacement boundary conditions are utilized as loads.

We updated the current Fig 1E with this contour plot of principal strain experienced by the membrane (Fig. 1). Certainly the strain variation outside of the imaging area can impact the response of the cells in the central area in a paracrine manner. There are multiple ways in which we intend to handle this in the future by designing wells to limit cell growth in this uniform area of strain. We have other alternatives that are in development towards enlarging the area of constant strain as well. We added comments to the latter part of the manuscript to address the issues regarding strain distribution.

Our aim in using the 4X markers was to show that the membrane stretches with the motor counts. Next, we utilized sparsely seeded cell nuclei at 20X or 40X to show that the cells indeed traveled with the membrane that shown to have stretched. We are in the process of carrying out research projects where we are observing deformations within a monolayer that are dependent upon an intact cytoskeleton within individual cells and inter-cellular connections. The tearing observed in phase contrast images in Fig. 6 at locations indicated by the arrows, and the way in which cells deform relative to each in the monolayer, are examples of local deformations within the cells. However, we have not quantified these local responses at this stage.

2. Availability of the device:
   We discussed this concern with JoVE editors as well. As a group of investigators who worked on this project, we understand the complexity in not only manufacturing this product but also using it. As a team, we are committed to sharing our design and expertise with those who are interested. We embarked on this design project since we did not believe that there were existing systems that could be modified to achieve our aims. Otherwise, as the first Reviewer suggests, it would have been a better choice for us to consider modification rather than a new design.

   At this time, we do not have detailed drawings, but we do have CAD models of the device and associated components.

3. The meaning of near real time:
   We addressed this comment by adding a description of what near realtime indicates in our work. What we intend to communicate is that the time between the end of stretch and image recording is less than 10 seconds. In the current version of the device, there is a minor xy- and out-of-plane plane travel of the region of interest. When using a low magnification objective, this becomes less of an issue, but with higher magnifications, there are occasions where the travel must be accounted for. This is why there can be a 10 second delay.

   There are three possible ways to improve this time limitation: 1) Redesign the system with tighter tolerances to limit xy-travel; 2) Utilize a motorized xy-stage to travel with the image as the device produces a repeatable xy-travel; 3) Utilize a motorized z-stage to account for the focus which consumes less of the time. As we develop the device further, we will take these into account and improve the time of image acquisition.

4. Lack of AFM results:
   We added a new section relating to the nano-indentation of the same field of MLE12 cells before and during stretch. In these experiments, we utilized live MLE12 cells and stretched them to 10% tensile strain. We recorded phase contrast images and generated elastic modulus maps similar to our previous work (Roan et al. 2012). We are conducting more experiments specifically designed to test hypotheses relating to our core research projects. In this manuscript, we presented examples of E-maps.

Response to Reviewers' Individual Comments

Reviewer #1:

Manuscript Summary:

In this article, the authors present a method and device for monitoring/imaging cell responses during static and cyclic mechanical stretch. Their device consists of a custom made motor-driven mechanical actuator that induces deformation of an elastic substrate. Many similar cell-straining devices have been developed in the past including commercially available systems (Flexercell) and publication of this established method appears appropriate for the JoVE format. However, this reviewer does have several concerns that should be addressed prior to publication. In particular, it should be clarified if this device can be used for long-term, real-time measurements as opposed to short-term "near" real-time measurements. In addition, I recommend removing the AFM portions of this manuscript since no data are presented in this regard and there are several questions about how this type of AFM measurement would be accomplished in moving/deforming cells.

Major Concerns:

1. One major concern I have is with the strain field generated by the device. Computational model results (Fig 1f) indicate that the device likely produces uniform strain fields but it also seems to generate significant spatial gradients in strain. Since strain gradients have been shown to be an important stimulus of cell behavior (Balestrini et al., Biomech Model Mechanobio, 9(3):329-344, 2010), the authors should discuss how they would either modify the device to minimize strain gradients or restrict cell growth/analysis to a region in which strain gradients are small. A related issue is that in Figures 2 and 3, the authors use fiducial markers, the cell body and the cell nucleus to measure global strain. However, these measurements do not provide information about the local strain field at the cellular level. Perhaps the authors could evaluate the magnitude of local strain by measuring changes in cell/nucleus shape?

   Please see Common Response 1 above.

2. My second major concern is that although the authors claim that they have developed a protocol for conducting AFM nano-indentation measurements on stretched cells, no data in this regard was provided. In addition to questions about how generalizable this system is given the highly specialized nature of the particular AFM system being used, there are several questions about how this measurement would be conducted. For example, it is unclear how the AFM indentation measurements would be performed on deforming/moving cells? This measurement would be particularly difficult given the need to move the AFM head/tip with the exact same motion as the underlying cell. Also, what were the device design considerations/modification needed given the tight clearance typically required for the AFM cantilever to avoid false engagement issues? Given that no representative results were presented for AFM, it might be easiest to remove this portion of the manuscript.

   Please see Common Response 4 above.

Minor Concerns:

1. The authors state that the device can be "placed in a cell culture incubator." However, the size of the current device likely prevents it from being integrated into a stage top incubator? This limitation will effect the ability of this device to continuously monitor cell responses by imaging over long periods of time (i.e. days).

   The device is compact, less then 45mm in height, fits into a cell incubator, and is driven by a cable. As such, it will not require any special accommodations. All of the parts can be sterilized. In addition, we have checked into some of the benchtop systems of environmental control. There are stage top systems that will accommodate our stretching device (http://www.wpiinc.com/products/physiology/iv-200-ox-microscope-environmental-chamber-heat-controller-co2-and-o2-controller/?back=products). The AFM itself is housed in a large environmental chamber, which can be supplied with gasses and also the AFM stage can be heated. We do not have this heated plate option at this time, but with minor modifications we are confident that we can implement our device on the heated petri dish holder provided by the AFM manufacturer.

2. Since the current device is custom made the authors should describe if the current technique is limited only to investigators with access to such a device or if any of the standard cell stretching devices could be modified to accomplish similar experiments.

   Please see Common Response 2 above.

3. Why did the authors use a pro-inflammatory cytokine pre-treatment condition when investigating the effect of stretch on gap formation? Can these gaps develop with mechanical stretch alone?

   In our research, we aim to link stretch and epithelial cell damage. Without a prior insult, such as a pro-inflammatory cytokine, we have not observed a tear within the 20% strain range with a *single* stretch. Otherwise, a persistent injurious cyclic stretch is needed to show damage in control monolayers as we have done in prior studies. In more recent work, our aim has been to capture in real time the tearing of cells and therefore we wanted to create a more clinically relevant condition such as pretreatment with TNF-a.

4. In several locations the authors reference "near" real-time measurements - they should clarify what they mean by this.

   Please see Common Response 3 above.

Reviewer #2:

Manuscript Summary:

Rapalo et al. demonstrate technique for biaxial stretching of cells on an elastic cell culture substrate using a novel design that allows for near-real time imaging.

Major Concerns:

1. The written document seems sufficient to reproduce the experiments if one has this exact device, but not to produce the device itself. Therefore the protocol may be of limited value. Could the device, including the cell stretching apparatus and motor/driver assembly, be described in sufficient detail (including detailed design drawings?) so that one could fabricate the entire system?

   Please see Common Response 2.

2. Since the main feature of the device is the design to keep the cells in plane during stretch, more attention should be given to this capacity and its limitations. Why is the imaging only "near real-time"? Must the microscope be refocused during/after stretch? Could a controllable focus be integrated with the device to track the cells during stretch in a future iteration of the device?

   Please see Common Response 3.

3. It is not clear whether the strain pattern is uniform across the surface and matches the FEM predictions.

   Please see the Common Response 1.

4. It would be helpful to more systematically compare the capabilities of this device to other biaxial stretch systems reported in the literature.

   We refrained from doing a systematic review due to limitations in the length of the manuscript.

Minor Concerns:

1. In the introduction, it would be more appropriate to list "the increase in reactive oxygen species (ROS)8,9 and pro-inflammatory cytokines" as an outcome of mechanotransduction (LINE 66).

   We changed the manuscript according to the reviewer's suggestion.

2. Also in the introduction, LINE 69 need a "the" in "Although the focus..."

   We changed the manuscript according to the reviewer's suggestion.

3. In the protocol, it is not clearly specified what material is used for the "flexible

membranes" and whether these are already coated with collagen. Details are in the table, but would be helpful in the protocol as well. Are there alternatives?

We would like to point out that in step 1.1, it is mentioned that they are coated with Collagen 1. We intentionally remained vague on this since there are alternatives to flexible membranes and coatings depending on cell type. However, we utilized the commercially available FlexCell membranes, which are made out of PDMS. We measured the elastic modulus of these membranes and they are in line with PDMS membranes that we also constructed in house.

4. The procedures listed in the protocol should better reference the figures to help explain each step. In addition, a schematic showing the steps used to produce the flexible membrane with attached wall would be helpful.

   We have addressed this throughout the manuscript and the protocol.

5. It is not clearly described whether the device can produce cyclic stretch, and what the limits are on the frequency, strain rate, etc.

   A motor that is capable of driving the system with various wafeworms and frequencies drives the device. In reality, we purchased a motor that is much more capable than our current demands (Smart Motor, 3400 Series): Nominal power 0.24 HP, Peak torque 1.06 N m. One of the many reasons that led us to buy this motor was that, with a simple USB interface, a simple code can be uploaded to drive the motor in whichever way the user desires.

6. The "mitochondrial superoxide indicator" is not described in sufficient detail in the protocol (LINE 207); though it is listed in the table it would be helpful here.

   The JoVE limits our use of specific brand information in the manuscript. However, it is, as the reviewer noted, available in the Material's list.

7. More detail should be given about the particular cells used in the experiments (LINE 311).

   We added details about the cells to the Materials List provided by JoVE.

Reviewer #3:

Manuscript Summary:

I find this to be a well written manuscript with clear and useful illustrations. I think with some clarifications and additions to the text, I would be able to achieve the types of results that are shown in figures 5 and 6. Though, of course, I would have to pay very close attention to the associated video. I have some questions and specific comments that might help the authors increase the clarity of the protocol.

Major Concerns:

1. No details have been provided regarding how to build the custom motor-driven mechanical actuator. Since this device is not commercially available and is required for the described methodology, knowing how to construct it seems like essential information.

   Please see Common Response 2.

2. In several places in the text, the author's language implies that confocal and atomic-force microscopy can be used simultaneously to image cells within the stretching device. However, in the discussion it is clear that the device has only been used with confocal or AFM, but not both together. I think the text (both the protocol and discussion sections) needs to accurately reflect this reality.

   Please see Common Response 4 above.

3. In several places the authors give vague instructions. This may be because the precise actions/settings depend on the equipment available. In these cases, I think the authors should at least state the equipment and settings they used (eg. steps 4.7 and 4.8)

   We understand the concern brought up by the reviewer. The equipment utilized is provided in the Materials List provided by JoVE. The settings are left vague in the protocol as these are highly dependent on the specific equipment.

Minor Concerns:

Abstract/Introduction

1. In the long abstract (line 53), there appears to be a typo with the word 'in' missing.
   We were unable to identify the missing position of the word “in”.

2. Is the excessive tensile strain or overdistention applied by the authors relevant to a pathological condition?

   Overdistention injury in the lung epithelium is one of the mechanisms by which overall damage worsens. We added clarification later in the document as per the reviewer’s comment.

3. What is the definition of tensile strain? Is it simply another way of saying 'stretch'?

Certainly, the reviewer is correct in equating tensile strain and stretch. However, in mechanics, the term strain is utilized when materials are deformed and tensile strain is the proper term. The issue is that there is another definition of deformation that is stretch ratio commonly utilized in nonlinear mechanics. As such, we intended to keep our terminology in line with mechanics.

Protocol

1. 1. What are the dimensions of the die punch?
   In the new version, we refrained from using die punch. We provided dimensions in Fig. 1E towards this concern. The die simplifies the cutting of the membrane into a desired, but this can also be achieved with a scalpel.

2. 3.2 What is the diameter of the 50 ml tubes?
   The inner diameter of the tube varies with the brand utilized. We have used 50mL tubes with an approximately 28 mm inner diameter.

3. 4. Are the membrane and PDMS ring inside a petri dish?
   During this experiment, the membrane and the ring is not in the petri dish. The wall of the membrane maintains DMEM for the cells.

4. 3.1 What is the source of the MLE12 cells?
   We added the cells in the Materials List. The cells were purchased from ATCC (Manassas, VA).

5. 3.2.2 This is the first mention of the tabs in the membrane. A clearer description of the membrane shape in step 1.1 would be helpful for this step as well.
   In Fig. 1D, we labeled the "Clamp Tabs". We added "see Fig. 1D" to this step.

6. 4.1 How much of the mitochondrial superoxide indicator is added to the DMEM?
   A final concentration of 5um was achieved and 1-2mL of DMEM was added. We refrained from providing an exact volume since stock solution concentrations may differ.

7. 4.7 Please indicate objective, lasers, camera, acquisition settings, timecourse settings necessary to achieve the data shown in Figure 5.

   We added a limited amount of information to the Figure caption rather than to the protocol. We limited this information because there may be differences between systems. The device will not limit the use of most upright objectives. However, issues similar to imaging through a plastic dish of 1mm thickness will be present when using inverted systems where 60X or above magnification is needed.

8. 4.8 What software, image processing and quantification was necessary to get the results shown in figure 5?

We utilized simple analyses tools provided in ImageJ. We added ImageJ to the materials list.

9. 5. In the note it says these steps are provided for a specific combination of AFM and optical microscope as shown in figure 4. What are these microscopes? Figure 4 only shows the AFM.

   Each AFM is unique and it can be placed on a limited number of microscopes based on system configuration and weight limits. Our AFM uses an Olympus IX71 microscope that is listed in the Materials list provided by JoVE. This is an inverted microscope and we have 10X, 20X, and 40X capabilities.

10. 5.1 This step is unclear.

    These are details that are relevant to the AFM measurements. We added axis information to the step and also the images. We modified the protocol to add clarity.

11. 5.2 This step is unclear.
    More information is added and steps revised.

12. 5.2.3 Can the specific details (objective, spacer size, and AFM set-up) used by this group be provided? Otherwise, this is quite unclear.

    We are in agreement with this reviewer. We have included some of this information in the Materials list, as we are not allowed to give model information in the text. We are also limited in how much explanation we can provide.

13. 5.2.6 This step is unclear.
    No change made in the protocol.

14. 5.2.7 This step is unclear.
    Minor changes implemented.

15. 5.2.8 What is the light source?
    There are multiple light sources: AFM top illumination and the optical microscope light source. Again, this may be dependent on the set-up.

16. 5.3.1 What is the "load-cell"?
    A load cell is the holder of the chip with the cantilever beam on which the laser is focused. This is a common term among AFM manufacturers.

17. 5.3.4 This step is unclear.
    The adapter is visible in Fig. 5A and labeled.

18. 5.4.3 What were the parameters used in this experiment?
    This detail is provided in the new section added for Nano-indentation of MLE12 cells and also in the caption of the Figure 5.

Reviewer #4:

Manuscript Summary:

This manuscript describes an interesting device that can potentially generate novel data in the cellular physiology of mechanotransduction. The major advantage of the proposed device is the ability for time-lapse imaging of the cells while they are stretching. Unfortunately, there are some problems in characterization of the stretch, in providing evidence of feasibility of AFM imaging, and in the quality of illustrative examples provided by the authors.

Major Concerns:

1. Figure 1F demonstrates that the mechanical strain is likely to be non-uniform in the radial direction. It is not clear why the authors did not compare this prediction with the actual measurements of radial non-uniformity that could be derived from imaging of "dot-labeled" membranes as in Figure 2B.

   Please see Common Response 1.

2. Figure 4. At least one successful example of AFM imaging of the cells before and after stretch has to be provided. I do have a concern that the stiffness of a cantilever may be comparable or even larger than the bending stiffness of the membrane. It is yet to be determined whether AFM could still work in these conditions.

   Please see Common Response 4 above.

3. Why the acquisition rate of ROS measurements is so small (less than one frame in 10 minutes)? If for some reason, the images cannot be acquired at a faster frame rate, the applicability of this device is greatly diminished. The whole point of a live cell imaging under the stretch is to record the kinetics of the ROS increase. This kinetics is completely lost in the figure 5 due to slow acquisition rate. Time lapse imaging with a higher rate has to be provided superimposed on the actual (measured but not modelled) changes of the axial strain.

   Please see Common Response 3.

4. Figure 5: It is not clear whether the observed "jump" of fluorescence is the increase in ROS production but not a mechanical artefact. For example, stretching and flattening of the cell may bring some out of focus fluorescence into the focal plane, which would increase the overall brightness of the signal. Perhaps, the authors need to provide the time course of fluorescence signal after unloading, which would be measured simultaneously with the recovery of the mechanical strain.

   We are confident that the results of our ROS measurements are not due to mechanical artifacts for the following reasons. More information can be found in the following abstract: Rapalo, Gabriel, et al. "Stimulation of mitochondrial superoxide in lung epithelial cells using a novel stretching device (869.5)." *The FASEB Journal* 28.1 Supplement (2014): 869-5.

1. We have carried out control experiments on static glass and plastic dishes. We did not observe a jump. We did not include these results in this manuscript.
2. We have carried out experiments with Cytochalasin D (actin disruptor) where the signal does not show a jump. We did not include these results in this manuscript.
3. We have inhibited the production of ROS with Tiron (ROS scavenger) in experiments with stretch and did not observe a jump in the signal.
4. In addition, the 16HBE cells are reasonably flat which reduces the out-of-plane effects significantly.

5. Figure 6: It is hard to determine whether the appearance of the gaps between the cells is due to the loss of cell-to-cell contacts or secondary to the detachment of the cell from the underlying membrane. As such, this is not a good model of "epithelial damage". Presentation of this crude experiment as a good model could be misleading.

   We understand the reviewer's concern as to whether the failure of cell-to-cell junctions were driven mainly by the cell adhesion deficiencies to the substrate or due to cell cytoskeletal changes. In our group, we have published work relating to the hypothesis that the formation of gaps between the cells with stretch in the presence of a biochemical stimulus is dependent on the cell cytoskeletal state. The idea is that the cells are more vulnerable to damage due to stiffening, as they are less likely to deform with stretch (Roan et al. 2012, Wilhelm et al. 2014). We would like to emphasize that Fig. 6(C-D) were obtained in a series of photographs recorded within less then a minute as we increased the stretch. One stretch led to opening of gaps and when we returned to the initial unstretched condition, we did not observe the tear in the monolayer to be repaired by the cells in that location. Moreover, we have many images where the cell occupying the empty area is still attached to one side of the cell-free area. We are working to elucidate the underlying mechanisms of this type of failure in the cell monolayer as we develop tools and protocols that enable us to undertake the experiments required to test our hypothesis.

Minor Concerns:
N/A

Reviewer #5:

Manuscript Summary:

In their manuscript, Rapalo and colleagues describe a new imaging protocol for visualization of cell-monolayers during mechanical stimulation. Their method is interesting and the manuscript is well written.

However, I have a few comments:

Major Concerns:

1. It is stated in the Introduction and in the Discussion sections that the strain created in the device is isotropic and homogeneous. However, comparison of the results of the FEM analysis (figure 1F) with figure 2 C indicates a certain discrepancy. The FEM analysis suggests a very homogeneous, nearly circular distribution of the strain. By contrast, in figure 2C a clear hexagonal deformation of the membrane is observable. My impression is that the strain on the real membrane is less homogeneous than the FEM analysis suggests.

   Please see Common Response 1.

2. In this context it has to be mentioned that the further analysis of strain is based on an analysis along three axes of same angle, thus similar strains rather result from the hexagonal structure of the setup than representatively reflect the homogeneity of the membrane's strain. For investigating the membrane strain it would be appropriate to investigate the strain curves along axes independent of the setup's geometry.

   Please see Common Response 1. We also want to note that, we added a discussion about the fact that the membrane design is in progress and that a uniform field can be attained with simple design improvements.

3. As the analysis of the cell's mechanics is addressed, for the sake of completeness, I would suggest to mention recent developments on this topic to the introduction section, e.g. Dassow et al. J Biomed Mater Res B Appl Biomater. 2013 Oct;101(7):1164-71 and Gamerdinger et al. J Mech Behav Biomed Mater. 2014 Aug 22;40C:201-209.

   We added the recommended papers to the manuscript's introduction.

4. Appropriate manufacturer information should be given for the used materials, i.e. linear motor system, software, PDMS, membrane material, collagen, microscope, AFM, etc.

   These are located in the Table provided by the JoVE. We are not allowed to include this information in the protocol.

Minor Concerns:

1. Protocol, point 3.2.2: wouldn't it be easier to punch the holes earlier, i.e. before

seeding the cells (e.g. as point 1.7)?
Addressed in the manuscript.

This is true and we have now incorporated the holes in the die punch. However, the 1mm holes are at the lower end of what can be handled with reasonable manufacturing complexity and low cost.

2. Line 149: '(10S:1 ratio)' should be '(10:1 ratio)'
Addressed in the manuscript.