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.

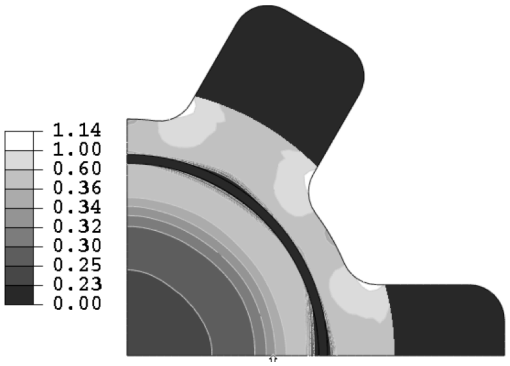

Fig. 1: Maximum principal strain distribution. Dark gray areas belong to the clamped regions. There are stress concentrations near the clamps that do not reach the center of the membrane. Quarter of the membrane is considered for finite element modeling, quad elements are utilized predominantly, and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