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Title: Equibiaxial Stretching Device for High Magnification Live-Cell Confocal Fluorescence Microscopy

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to takef place in multiple locations? **No**

Current Protocol Length
Number of Steps: 18
Number of Shots: 54 (19 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Videographer's NOTE:
J25-003_ID#67520 //
01 INTWs (4) >> 4 files
02 PROTOCOL (44) >> 44 files
03 HEADSHOTS (3) >> 3 files
04 NOTES (1) >> 1 file
05 LUTs

REQUIRED:

1.1. **Anabel-Lise Le Roux:** We study how cells respond and adapt to mechanical stimuli in physiological and pathological context. For this we use the stretching device to apply external forces, a strain in this case, to cells.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.4*

What significant findings have you established in your field?

1.2. **Manuel Gomez:** We have found how cells and cell tissues react to specific mechanical stimuli, and how they mechanically respond to other biological cues.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.1*

What advantage does your protocol offer compared to other techniques?

1.3. **Valeria Venturini :** Our stretching device is very versatile compared to others as it offers the possibility to stretch cells cultured on polyacrylamide gels of different stiffnesses or to perform compression experiments.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions:

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.4. **Anabel-Lise:** We diffuse a full protocol of our custom-made stretching device to the community, showing its reliability and versatility and how to build it from zero. We hope the community can benefit from this compiled information while becoming aware of the many use of the device, to advance research in cell mechanobiology.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.4*

Protocol

2. PDMS Membrane Sandwiching in Stretch-Rings

Demonstrators: Valeria Venturini, Anabel-Lise Le Roux

2.1. To begin, place the PMMA rings on a sheet of white bench paper **[1-TXT]**. Carefully go around the edge of the PDMS-coated plate using a blade to ensure a clean cut along the entire circumference **[2-TXT]**. Use tweezers to lift the membrane at one edge, then gently peel it with the thumb to evenly detach half of the membrane from the plate **[3]**.

2.1.1. WIDE: Talent placing the PMMA rings placed on white bench paper. **TXT: PMMA: Polymethyl Methacrylate**

2.1.2. Talent using a blade to cut along the PDMS edge. **TXT: PDMS: Polydimethylsiloxane**

2.1.3. Talent peeling and lifting the membrane evenly along half of the plate circumference.

2.2. Use fingers to spread 96% ethanol evenly around the entire top surface of the PMMA ring **[1]**. Carefully remove the PDMS membrane from the plate by lifting it from the previously lifted edge **[2]**, then place it symmetrically onto the ethanol-covered PMMA ring, ensuring no wrinkles while avoiding stretching **[3-TXT]**.

2.2.1. Talent wiping ethanol on the PMMA ring with fingers.

2.2.2. Talent lifting the PDMS membrane from the previously lifted edge. NOTE: 2.2.2 and 2.2.3 are merged.

2.2.3. Close-up Shot of placing the membrane onto the ethanol-covered ring without wrinkles. **TXT: Let the ethanol dry; PDMS membrane adheres to the PMMA ring**

2.3. Then, take another empty PMMA ring and place it on top of the mounting ring **[1]**. Use metal clips to seal them together, sandwiching the PDMS membrane between the two rings **[2]**. Position the bottom half of the stretch ring onto the mounting support **[3]**. Place the PDMS ring sandwich onto the stretch ring and push it down firmly until fully seated, ensuring the gasket is correctly positioned **[4]**. Place the circular brass weights on top of the sample **[5]**.

2.3.1. Talent placing an empty PMMA ring over the mounting ring.

2.3.2. Talent securing the rings together using metal clips.

2.3.3. Talent positioning the stretch ring onto the mounting support.

2.3.4. Talent pressing the PDMS ring sandwich into the stretch ring, ensuring the gasket is aligned.

2.3.5. Talent placing circular brass weights on top.

2.4. Next, place the top half of the metal stretch ring on top, aligning the screw holes **[1]**. Insert all screws and tighten them **[2]**. Cut the PDMS around the ring gently using a blade **[3]**. Cover the assembly with a Petri dish to protect the membrane from dust **[4-TXT]**.

2.4.1. Talent aligning the top half of the stretch ring and screw holes.

2.4.2. Talent inserting and tightening the screws.

2.4.3. Talent cutting excess PDMS around the ring with a blade.

2.4.4. Talent covering the setup with a Petri dish to prevent dust contamination. **TXT: Attach a polyacrylamide (PAA) gel on top of the PDMS membrane**

3. Stretching Device Operation, Strain Calibration and Resolution Assessment

Demonstrators: Manuel Gomez, Anabel-Lise Le Roux

3.1. Prepare a 1 to 50000 dilution of 0.2-micrometer fluorescent beads in PBS using serial dilution **[1]** and place a 10 to 30 microliter drop of the bead solution at the center of the PDMS **[2]**. Allow it to dry at a temperature no higher than 40 degrees Celsius to avoid post-curing confounding issues **[3]**.

3.1.1. Talent mixing the fluorescent bead with water.

3.1.2. Talent pipetting a drop of the bead solution onto the PDMS center.

3.1.3. Close-up of the PDMS membrane being placed in an incubator.

3.2. Now, mount the stretching device onto the microscope stage holder and secure the stretching post onto the microscope **[1]**. Generously apply lubricant to the circular area of the post **[2]**. Mount the prepared stretch ring onto the stretching post and screw the top cap to seal the assembly **[3]**. Connect the vacuum controller to the vacuum source and the stretching post **[4]**. Then, connect the rear of the vacuum control box to the vacuum source followed by the front of the box to the stretch post **[5]**.

3.2.1. Talent securing the stretching post on the microscope.

3.2.2. Talent applying lubricant to the circular area of the post.

3.2.3. Talent mounting and sealing the stretch ring assembly.

3.2.4. Talent connecting the vacuum controller to the vacuum source and stretching post. NOTE: 3.2.4 and 3.2.5 are combined.

3.2.5. Close-up of the vacuum controller connected to source and stretch post.

3.3. To perform strain calibration, choose a field of interest containing a high bead density and recognizable bead clumps for the user **[1]**. Acquire an image at rest of the selected field of view and save the position **[2]**. Apply stretch at a low vacuum level of negative 20 millibar, causing movement in all directions **[3]**. Now, refocus in the Z-plane *(zee-plane)* and move the stage in X-Y directions to locate the same region of interest and acquire an image in the stretched state **[4]**.

3.3.1. SCREEN: 67520_screenshot_1 0.03-00.10.
3.3.2. SCREEN: 67520_screenshot_1 00:11-00:20.
3.3.3. SCREEN: 67520_screenshot_1 00:45-00:59.
3.3.4. SCREEN: 67520_screenshot_1 01:55-02:10.

3.4. Then, release the strain and repeat the steps with a higher vacuum level **[1-TXT]**. Store acquired images in a folder organized by sample **[2]**. Name the reference bead image acquired without stretch as **reference_str1** *(reference-S-T-R-1)* and the images stretched at increasing vacuum levels as **stretched_str1, stretched_str2, stretched_str3** *(stretched-S-T-R-1-2-3)* and so on **[3]**.
3.4.1. SCREEN: 67520_screenshot_1 02:20-02:30. **TXT: Repeat the strain calibration steps for at least 3 different stretch rings**
3.4.2. SCREEN: 67520_screenshot_2 00:02-00:04.
3.4.3. SCREEN: 67520_screenshot_2 00:06-00:28. *Video editor: Please speed up if required*

3.5. To quantify the strain, open MATLAB and load the **gel_strain** *(gel strain)* code **[1-TXT]**. After setting the parameters, define the parent as well as the experiment folder paths and run the code **[2]**.
3.5.1. SCREEN: 67520_screenshot_2 000:30-00:40. **TXT: https://github.com/xt-prc-lab/Le_Roux_et_al_2024_JOVE**
3.5.2. SCREEN: 67520_screenshot_2 00:41-00:55.

3.6. Then, open stretched_str1 *(stretched-S-T-R-1)* as requested by the code, followed by the reference_str1 **[1-TXT]**. Now, calculate the median strain matrix for each vacuum level. After repeating this process for at least three samples, plot the median strain matrix versus vacuum level to obtain the calibration curve **[3]**.
3.6.1. SCREEN:67520_screenshot_2 00:56-01:05. **TXT: Verify that the results folder appears with 4 subfolders**
3.6.2. SCREEN: 67520_screenshot_2 07:00-07:14.

4. Cell Seeding, Stretching and Image Acquisition

4.1. To coat the PDMS ring, sterilize it under ultraviolet light for 15 minutes **[1]**. Under a biosafety cabinet, rinse the plate under the hood with sterile PBS **[2].** After aspirating all the PBS used to rinse the plate **[3]**, deposit a 100 to 200-microliter drop of the solution in the center of the ring **[4]**. Draw the perimeter of the drop with a permanent marker from the bottom side of the PDMS membrane and incubate it **[5]**.
NOTE: The VO has been edited.
4.1.1. Talent placing the PDMS ring under ultraviolet light for sterilization.
4.1.2. Talent adding the PDMS membrane with sterile PBS.
Added shot: 4.1.2.Extra: Talent aspiring all of the sterile PBS from the ring.

4.1.3. Close-up Shot of pipetting a cell adhesion protein drop onto the membrane.
4.1.4. Close-up of the marker-drawn perimeter around the drop.

4.2. On the day of the experiment, wash the adhesion protein with PBS **[1]**. After trypsinizing the cells, proceed to cell counting **[2]**. Seed an appropriate number of cells in a small volume of about 20 to 50 microliters with standard cell media to promote attachment **[3]** and incubate it in a carbon dioxide incubator with humidity control **[4]**. NOTE: The VO has been edited.

Added shot: Talent washing the adhesion protein with PBS. Author's NOTE: Named Talent 4.1.3extra but it is to be placed before 4.2.1.

4.2.1. Talent placing the sample in a cell counter. **TXT: If needed, transfect the cells of interest with fluorescent protein beforehand**
4.2.2. Talent pipetting 20–50 microliters of cells onto the PDMS ring.
4.2.3. Talent placing the sample in an incubator.

4.3. After 20 to 30 minutes, gently add 500 microliters of media on top of the cells **[1]** and return the plate to the incubator to allow for further cell spreading **[2-TXT]**.
4.3.1. Talent adding 500 microliters of media after incubation.
4.3.2. Talent placing the sample in an incubator. **TXT: Replace the spent medium with CO_2-independent media**

4.4. For a single stretch-release cycle and imaging, mount the stretch ring onto the microscope stage as demonstrated earlier **[1]**. After locating a cell of interest, record its position and capture an image of the pre-stretched cell **[2]**. Apply the desired vacuum level **[3]** corresponding to the required substrate strain based on the calibration curve to stretch the cell **[4]**. NOTE: The VO has been edited.
4.4.1. Talent mounting the stretch ring onto the microscope stage.
4.4.2. SCREEN: 67520_screenshot_3-(1).avi 00:17-00:25 and 00:34-00:35.
4.4.3. Talent applying vacuum by turning the vacuum controller ON.
Added shot: 4.4.3.Extra: Talent showing how the PDMS membrane is sucked by vacuum and deforms.

4.5. To locate the stretched cell, first refocus in the Z-plane, then move in the X-Y directions to center the cell in the field of view **[1]**. Record images during the desired duration **[2]**. Navigate to the cell's original position and activate the microscope's acquisition mode to ensure that image capture begins immediately upon refocusing **[3]**. Release the stretch **[4]** and quickly refocus manually in the Z-plane **[5]**. Capture images of the released cell for the required time, manually adjusting the focus throughout the acquisition **[6]**.
4.5.1. SCREEN: 67520_screenshot_3-(1).avi 00:49-00:52 and 00:56-01:00.
4.5.2. SCREEN: 67520_screenshot_3-(1).avi 01:05-01:10.
4.5.3. SCREEN: 67520_screenshot_3-(1).avi 01:10-01:25.

4.5.4. Talent releasing vacuum by turning the vacuum controller OFF
4.5.5. SCREEN: 67520_screenshot_3-(1).avi 01:27-01:35.
4.5.6. SCREEN: 67520_screenshot_3-(1).avi 01:45-01:55.

4.6. For post-processing, open Fiji and load the images acquired at rest and during strain **[1]**. Use the Fiji plugin template matching to select a region of the cell for alignment **[2]**.
4.6.1. SCREEN: 67520_screenshot_4-(1).mp4 00:10-00:20.
4.6.2. SCREEN: 67520_screenshot_4-(1).mp4 00:35-00:45.

Results

5. Results

5.1. Calibration ensured reproducibility and control over applied strain, confirming a nearly linear relationship between vacuum and strain up to 20% **[1]**.

5.1.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the calibration curve showing the strain increasing with vacuum*.

5.2. Radial displacement analysis demonstrated isotropy and homogeneity of strain over the PDMS membrane **[1]**.

5.2.1. LAB MEDIA: Figure 3B. *Video editor: Mark the row showing "radial displacement" distribution*.

5.3. Fluorescence imaging of fibroblast cells under live-cell stretching revealed membrane evaginations upon stretch release **[1]**, which reabsorbed within 3 to 5 minutes **[2]**.

5.3.1. LAB MEDIA: Figure 4B and C. *Video editor: Highlight the magenta-stained border in C for column "Stretch (magenta) versus initial state (green)".*

5.3.2. LAB MEDIA: Figure 4B and C. *Video editor: Highlight the magenta-stained border in C for column "Stretch (magenta) versus relaxed state (green)".*

5.4. Restretching of fixed cells enabled imaging in a stretched state, preventing artificial folding artifacts **[1]**.

5.4.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the image labeled " Restretched fixed cells"*.

5.5. PDMS membrane with a patterned grid facilitated multi-modal imaging, allowing localization of the same cells in fluorescence and scanning electron microscopy **[1]**.

5.5.1. LAB MEDIA: Figure 4F.

5.6. Soft PAA gel coatings influenced cell adhesion and spreading under cyclic stretch, with fibroblasts forming focal adhesions on stiff substrates but remaining rounded on soft ones **[1]**.

5.6.1. LAB MEDIA: Figure 4E.

Pronunciation Guides:

1. Trypsinizing

Pronunciation link:
https://www.merriam-webster.com/medical/trypsinize
IPA: /ˈtrɪpsəˌnaɪzɪŋ/
Phonetic Spelling: trip-suh-nye-zing

2. Evaginations

Pronunciation link:
https://www.merriam-webster.com/dictionary/evagination
IPA: /ˌiːvædʒəˈneɪʃənz/
Phonetic Spelling: ee-vaj-uh-nay-shuhnz

3. Fluorescence

Pronunciation link:
https://www.merriam-webster.com/dictionary/fluorescence
IPA: /ˌflʊəˈrɛsəns/
Phonetic Spelling: floor-ess-ens

4. Peristalsis

Pronunciation link:
https://www.merriam-webster.com/dictionary/peristalsis
IPA: /ˌpɛrəˈstɔːlsɪs/
Phonetic Spelling: pair-uh-stawl-sis

5. Cyclic

Pronunciation link:
https://www.merriam-webster.com/dictionary/cyclic
IPA: /ˈsaɪklɪk/
Phonetic Spelling: sigh-klik

6. Pipette

Pronunciation link:
https://www.merriam-webster.com/dictionary/pipette
IPA: /paɪˈpɛt/
Phonetic Spelling: pie-pet

7. Micropipette

Pronunciation link:
https://www.merriam-webster.com/dictionary/micropipette
IPA: /ˌmaɪkroʊpaɪˈpɛt/
Phonetic Spelling: my-kroh-pie-pet

8. Transfect

Pronunciation link:
https://www.merriam-webster.com/dictionary/transfect

IPA: /trænˈsfɛkt/
Phonetic Spelling: tran-sfekt