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Title: Controlled Strain of 3D Hydrogels Under Live Microscopy Imaging

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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 similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y, all done**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **43**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Avraham Kolel**: Biological tissues grow in 3-dimensional soft environments, through which cells are exposed to various mechanical cues. Our method allows the stretching of hydrogels in a 3-dimensional manner for the investigation of biomechanical responses **[1]**.

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

REQUIRED:

1.2. **Avraham Kolel**: This technique enables the uniform stretching of hydrogels along their thickness while live imaging is performed. Additionally, the hydrogel's geometry can be manipulated to any size or shape **[1]**.

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

OPTIONAL:

1.3. **Ayelet Lesman**: Knowledge about how cells and the extracellular matrix respond to forces is important for revealing how tissues develop and diseases progress, leading to the potential development of cancer therapies and tissue engineering models **[1]**.

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

Protocol

2. Silicone Strip Preparation

2.1. Begin by using a laser or manual cutter to create a rubber strip with a hole in the center **[1]**. Cut a rectangle of hydrophobic film so that it is wider than the silicone strips **[2]** and wash a plastic dish with 70% ethanol **[3]**.

2.1.1. WIDE: Talent cutting pieces
2.1.2. Talent cutting layer(s)
2.1.3. Talent washing dish, with ethanol container visible in frame

2.2. After drying with lint free wipes, place the sealing film into the dish and place one silicone strip with the plastic wrap removed from one side into the center of each piece of sealing film **[1]**.

2.2.1. Talent placing film into dish + removing plastic and placing strip into dish

NOTE: Here two shots 2.2.1 and 2.2.2 were filmed as one shot and hence the VO description was also changed to 1 step.

3. Fibrin Gel Polymerization

3.1. For fibrin gel formation, uniformly deliver 2.5 microliters of cooled, labeled fibrinogen **[1]** into the silicone cut-out of each strip so that the entire circumference of each cut-out is in contact with fibrinogen, taking care not to allow any air-pockets or bubbles to form anywhere in the solutions **[2]**.

3.1.1. WIDE: Talent loading pipette with fibrinogen *Videographer: Important/difficult step*
3.1.2. Fibrinogen being added to cutout, with fibrinogen container visible in frame *Videographer: Important/difficult step*

3.2. Immediately add 2.5 microliters of cooled thrombin directly to each fibrinogen solution **[1]** and quickly mix the solutions with careful pipetting, moving the tip around the entire mixture to create as homogenous a solution as possible **[2]**.

3.2.1. Thrombin being added to fibrinogen, with thrombin container visible in frame *Videographer: Important/difficult step*
3.2.2. Mixture being pipetted *Videographer: Important/difficult step*

3.3. After mixing, place the covered dish in the incubator for 30 minutes for gel polymerization **[1]**.

3.3.1. Talent placing dish into incubator

3.4. At the end of the incubation, add enough PBS to the dish to submerge the gel-silicone constructs **[1]** and carefully lift each sample from the dish, making sure that the sealing film layer remains adhered to the strip **[2]**.

3.4.1. Construct being submerged
3.4.2. Sample being lifted

3.5. Slowly peeling from one end of the silicone to the other, carefully detach the sealing film from each piece of silicone **[1-TXT]**. Then place the strip back into the dish **[2]** and place the dish on the stage of a light microscope to assess the condition of the sample **[3]**.

3.5.1. Film being peeled from silicone *Videographer: Important step* **TEXT: Avoid pulling areas close to the cut-out where stress concentrations may exist**
3.5.2. Talent placing strip into dish
3.5.3. Talent placing dish onto stage

4. Sample Loading

4.1. To load the sample onto the stretching device, fill the bath with PBS **[1]** and place the silicone strip containing the sample gel across the top of the bath so the ends of the strip are sitting on each side of the bath **[2]**.

4.1.1. WIDE: Talent filling bath
4.1.2. Talent placing strip across bath

4.2. Place the clamps and fabric strips **[1]** and then tighten so that all of the pieces are connected to form one straight strip with the cut-out in the center **[2]**. Place the material on the well **[3]** and secure the coverslip on the bottom of the well **[4]**. Then, place an aluminum liquid well into the stretching device **[5].**

4.2.1. Clamps and strips being placed on strip
4.2.2. Clamps tightened
4.2.3. Talent Placing material on well, with material container visible in frame
4.2.4. Coverslip placed on well
4.2.5. Talent placing well into device

NOTE: In step 4.2, few shots were added by authors and hence, the step description was changed accordingly.

4.3. Fill the well with 1-2 milliliters of PBS/cell medium **[1]** and place the strip-fabric-gel construct into the device **[2]**. Clamp the fabric strip into the bracket so that the cut-out of the gel is in the center **[3]** before carefully placing and locking the pin-down insert into place in the device **[4]**.

4.3.1. Talent filling well, with solution container visible in frame
4.3.2. Talent placing construct into device
4.3.3. Talent clamping strip in bracket *Videographer: Important step*
4.3.4. Talent placing/locking down insert in device *Videographer: Important step*

4.4. Place and lock other fabric side into the spindle **[1]** and place the stretching device and the attached sample onto the stage of a confocal microscope **[2]**. Use a USB cable to connect the microcontroller to the computer **[3]** and connect the servomotor to the microcontroller **[4]**.

4.4.1. Talent locking fabric side into spindle
4.4.2. Talent place device onto microscope stage
4.4.3. Talent connecting microcontroller to computer
4.4.4. Talent connecting servomotor to microcontroller

NOTE: Note to video editor: Use only the beginning & end of shot in 4.4.4.

4.5. Then open the stretching device control module on the computer to image the sample **[1]**.

4.5.1. Talent opening module, with monitor visible in frame

5. Sample Adequacy Confirmation

5.1. To determine whether the sample is adequate for an experiment, use low-resolution live imaging to scan the entire gel **[1]** and determine the lowest *Z*-position at which full adhesion to the inner walls of the cut-out is apparent with no tears or bubbles **[2]**.

5.1.1. WIDE: Talent scanning gel, with monitor visible in frame
5.1.2. SCREEN: 61671_Screencapture_1.mp4. 0:18 – 0:39. *Video Editor: Speed this up*

5.2. After noting the *Z*-location of the microscope, scan the interface of the fluorescent label of the gel and silicone strip to determine the full adhesion of the gel to the silicone throughout its circumference **[1]**.

5.2.1. SCREEN: 61671_Screencapture_1.mp4. 1:08 – 1:17.

5.3. After scanning, move the stage in the *Z*-direction until there is no longer continuity in the gel and note the upper limit of the *Z*-position **[1]**. Then subtract the upper limit of the *Z*-direction from the lower limit to calculate the sample thickness **[2-TXT]**.

5.3.1. SCREEN: 61671_Screencapture_1.mp4. 2:02 – 2:10.
5.3.2. BLACK TEXT WHITE BACKGROUND: $Z_o = Z_u - Z_\ell$ *Video Editor: emphasize Z-U with "upper limit", Z-L with "lower limit", and Z-o with "sample thickness"*

6. Stretching Device Operation, Stretching, and Imaging

6.1. To determine the pre-stretched position of the sample **[1]**, click **Go To Zero Servo Pos** in the module software to make sure the servomotor is at its zero position **[2]** and attach the servomotor to the stretching device, taking care not to put any strain on the sample **[3]**.

6.1.1. WIDE: Talent clicking button, with monitor visible in frame
6.1.2. Close up of motor
6.1.3. Talent attaching servomotor to device

6.2. While imaging, click the **plus 1** button to move the motor one degree at a time in the clockwise direction. When the right side of the cut-out moves, click the **minus 1** button to reverse the sample to the penultimate position to maintain the sample under minimal tension **[1]**.

6.2.1. SCREEN: 61671_Screencapture_1.mp4. 4:34 – 4:42

6.3. Click **Set Min Servo Position** to set the reference position **[1-TXT]** and capture a 40x magnification, high resolution, single Z-slice tile image of the entire gel area as a baseline reference for the post-processing analysis **[2]**.

6.3.1. SCREEN: 61671_Screencapture_1.mp4. 4:42 – 4:46. **TEXT: Click Go To Min Servo Position to return to reference position at any time**
6.3.2. SCREEN: 61671_Screencapture_1.mp4. 16:12 – 16:32. *Video Editor: Speed this up*

6.4. When all of the images have been acquired, advance the servomotor one degree per second until the desired stretch magnitude is reached **[2]**.

6.4.1. SCREEN: Screencapture_1.mp4. 9:01 – 9:10.

6.5. At each stretch magnitude for which an analysis is desired, verify that the gel has not detached from the silicone at any point throughout its circumference by scanning the interface between the gel and the silicone **[1].** Then, capture a high-resolution tile *Z*-stack image set of the entire gel area for post-processing analysis **[2]**.

6.5.1. SCREEN: 61671_Screencapture_1.mp4. 16:59 – 17:05.
6.5.2. Use 6.6.1. *Video Editor: please emphasize red gel and black interface when mentioned*

Results

7. Results: Representative Gel Strain Stretching and Gel Fiber Alignment Analysis

7.1. Zooming in and manually tracking bead aggregates during gel stretching **[1]** allows calculation of the local gel strains in the axial and perpendicular directions **[2]**.

7.1.1. LAB MEDIA: Figures 9A and 9B *Video Editor: please zoom into the dashed square for one image from Figure 9A to show corresponding magnification from Figure 9B*
7.1.2. LAB MEDIA: Figures 9B and 9C *Video Editor: please keep showing zoomed image from 8.1.1., add Figure 9C, and emphasize Egel text and arrow*

7.2. Typically, the axial strains propagate relatively linearly from the silicone cut-out edge to the center of the gel and are larger than the compressive perpendicular strains **[1]**.

7.2.1. LAB MEDIA: Figure 9D *Video Editor: please emphasize red data line in Figure 9D*
7.2.2. LAB MEDIA: Figure 9D *Video Editor: please emphasize green data line in Figure 9D*

7.3. Here high-magnification images **[1]** of a typical un-stretched and relatively isotropic hydrogel **[2]** and a hydrogel under high 80% cut-out strain, depicting highly aligned fibers in the stretch direction, are shown **[3]**.

7.3.1. LAB MEDIA: Figures 11A and 11B
7.3.2. LAB MEDIA: Figures 11A and 11B *Video Editor: please emphasize Figure 11A*
7.3.3. LAB MEDIA: Figures 11A and 11B *Video Editor: please emphasize Figure 11B*

7.4. Analysis of the fiber reorientation **[1]** revealed an approximately linear dependence between the fiber alignment and the external strain on the cut-out up to strains of 40% **[2]**, with moderate saturation at strains above 40% **[3]**.

7.4.1. LAB MEDIA: Figures 11A, 11B, and 11D
7.4.2. LAB MEDIA: Figures 11A, 11B, and 11D *Video Editor: please emphasize data lines from 0-40%*
7.4.3. LAB MEDIA: Figures 11A, 11B, and 11D *Video Editor: please emphasize data lines from 40-80%*

7.5. As illustrated in this fiber alignment analysis **[1]**, as the external strain on the cut-out increases **[2]**, the fiber alignment increases, following an overall non-linear curve **[3]**.

7.5.1. LAB MEDIA: Figure 12
7.5.2. LAB MEDIA: Figure 12 *Video Editor: please sequentially emphasize top left graph and green data line together then bottom left graph and blue data line together*
7.5.3. LAB MEDIA: Figure 12 *Video Editor: please emphasize blue data point in Figure 12C*

7.6. Note that the gel response to stretch is relatively uniform along its Z-thickness **[1]**.

7.6.1. LAB MEDIA: Figure 12B *Video Editor: please emphasize the colored straight and horizontal lines of Figure 12B*

7.7. As illustrated in this preliminary finite element simulation performed on 2D continuous material **[1]**, the color-map ranges from 38-42%, indicating that the gel strains are relatively homogenous throughout the circular gel area **[2]**.

7.7.1. LAB MEDIA: Figure 13
7.7.2. LAB MEDIA: Figure 13 *Video Editor: please emphasize heat map distribution in image from center to top and bottom of circle*

Conclusion

8. Conclusion Interview Statements

8.1. **Avraham Kolel**: It is important to ensure the hydrogel completely adheres to the inner walls of the geometric cut-out, as adhesion and continuity is crucial for reliable stretching of samples **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *(3.1., 3.6., 5.1.-5.4.)*

8.2. **Avraham Kolel**: By modifying the geometry of the silicone cut-out we can program gradients in strain and fiber alignment, so when embedding cells, we can analyze their response to these gradients **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

NOTE: Conclusion statements were modified.