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Title: Traction Force Microscopy to Study B Lymphocyte Activation

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **YES**

    If **Yes**, can you record movies/images using your own microscope camera?

    Yes

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

3. **Filming location:** Will the filming need to take place in multiple locations? **No, different floors of the same building**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Paolo Pierobon:** This method makes it possible to measure the spatiotemporal distribution of the forces applied by B cells at the immune synapse and correlate them with the recruitment of specific proteins.

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

1.2. Anita Kumari**:** Traction Force Microscopy using polyacrylamide gels is easy to implement. This protocol can be used to quickly set up the measurement of the mechanical capabilities of many B cells.

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

OPTIONAL:

1.3. Anita Kumari**:** This method is flexible and can be adapted to graft other ligands like integrins, or to study other kinds of immune synapses like T cells or frustrated phagocytosis.

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

1.4. **Judith Pineau:** Due to the physical and chemical properties of the gel required by this experiment, the adaptation of classical traction force microscopy methods to B cells may be tricky.

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

Protocol

2. Gel Preparation

2.1. Begin by silanizing the gel support **[1]**. Activate the coverslip or glass-bottom Petri dish with a UV lamp for 2 minutes **[2]**, then silanize it with 200 microliters of APTMS for 5 minutes. This will prepare the support for the covalent binding of the gel **[3-TXT]**.

2.1.1. WIDE: Establishing shot of talent at the lab bench in front of the gel support.

2.1.2. Talent exposing the coverslip or Petri dish to UV.

2.1.3. Talent silanizing the coverslip and glass-bottom dish. **TEXT: APTMS: aminopropyltrimethoxysilane**

2.2. Thoroughly wash the coverslip or glass-bottom dish with ultra-pure water **[1]** and dry it using vacuum aspiration **[2]**.

2.2.1. Talent washing the coverslip or dish.

2.2.2. Talent drying the coverslip or dish.

2.3. To prepare the coverslips for flattening the gel, put them into a ceramic coverslip holder **[1]**, put the holder in a small beaker **[2]**, and pour siliconizing reagent over the coverslips, making sure to cover them completely **[3]**.

2.3.1. Talent putting the coverslips in the holder. Videographer NOTE: slated 2.2.2, see file name

2.3.2. Talent putting the holder in the beaker. Videographer NOTE: 2nd part

2.3.3. Talent pouring the siliconizing reagent over the coverslips.

2.4. Cover the beaker with aluminum foil and leave it at room temperature for 3 minutes **[1]**. Meanwhile, fill a large beaker with ultra-pure water **[2]**. After 3 minutes of incubation in siliconizing reagent, transfer the coverslip holder with the coverslips to the beaker with water **[3]**.

2.4.1. Talent covering the beaker with aluminum foil.

2.4.2. Talent filling a large beaker with water.

2.4.3. Talent transferring the coverslips to the water.

2.5. Thoroughly rinse the coverslips with ultra-pure water **[1]**, dry them well, and place them on paper wipes. For best results, immediately proceed with gel polymerization **[2]**.

2.5.1. Talent rinsing the coverslips with water.

2.5.2. Talent drying a coverslip and placing it on a paper wipe. NOTE: CU

2.6. Prepare a 500-pascal gel premix according to manuscript directions, then combine 167 microliters of the premix with 1.67 microliters of beads **[1]**. Vortex and sonicate the mixture in a bath sonicator for 5 minutes **[2]**. Protect the mix from light with aluminum foil **[3]**.

2.6.1. Talent combining the gel premix with the beads. Videographer NOTE: take 2

2.6.2. Talent putting the gel mix in the sonicator bath.

2.6.3. Talent wrapping the mix in aluminum foil.

2.7. To catalyze polymerization, add 1.67 microliters of 10% ammonium persulfate to the gel mix **[1]**. Then, initiate polymerization by adding 0.2 microliters of TEMED *(pronounce 'tea-med')* and mixing the gel with a pipette **[2]**. *Videographer: This step is important!*

2.7.1. Talent adding APS to the gel mix, with the APS container in the shot.

2.7.2. Talent adding TEMED to the gel mix and mixing it, with the TEMED container in the shot. Videographer NOTE: take 2, Cu mixing

2.8. To cast the gel, pipet 9 microliters of gel mix onto each silanized coverslip or glass-bottom dish **[1]**. Immediately flatten the gel with the siliconized coverslip, pressing down on it with forceps to ensure that the gel spreads across the entire area of the coverslip and that some of it leakes out **[2]**. *Videographer: This step is important!*

2.8.1. Talent pipetting the gel mix onto the coverslip or glass dish.

2.8.2. Talent covering the gel with the coverslip and pressing down on it with forceps.

2.9. Invert the coverslip or glass-bottom dish into a large Petri dish and tap it on the bench to force the beads towards the gel surface **[1]**. Place a humidified tissue on the dish to create a wet chamber, **[2]** cover it with aluminum foil, and incubate it for 1 hour **[3]**.

2.9.1. Talent inverting the coverslip or dish onto a large Petri dish and tapping it on the bench.

2.9.2. Talent covering the dish a tissue and wetting it.

2.9.3. Talent covering the dish with aluminum foil. Videographer NOTE: 2nd part

2.10. After the incubation, facilitate coverslip release by adding PBS to the sample **[1]**. Carefully remove the coverslip with a needle, slightly tilting the dish but making sure that the gel is submerged in the PBS **[2]**. *Videographer: This step is important!*

2.10.1. Talent adding PBS to the dish, with the PBS container in the shot.

2.10.2. Talent removing the coverslip.

2.11. **Anita Kumari:** The siliconizing agent, acrylamide and bis-acrylamide, and TEMED can be toxic by inhalation. Wear standard personal protective equipment and manipulate these products under a chemical hood.

2.11.1. INTERVIEW: Named talent says the statement above in an interview-style shot. Videographer NOTE: take 2 last one

3. Gel Functionalization

3.1. Aspirate the PBS from the gels **[1]** and add 150 microliters of Sulfo SANPAH *(pronounce as 'SUN-PA')* at room temperature **[2]**. Expose the gel to UV treatment for 2 minutes **[3]**, then wash the gel 3 times with PBS **[4]**.

3.1.1. Talent aspirating PBS from the gel.

3.1.2. Talent adding Sulfo SANPAH to the gel, with the Sulfo SAMPAH container in the shot. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 3.2.1.*

3.1.3. Talent exposing the gel to UV.

3.1.4. Talent washing the gel with PBS. NOTE: CU + MED

3.2. Repeat the treatment with Sulfo SANPAH and the PBS washes **[1]**, then add 250 microliters of hen egg lysozyme, or HEL *(spell out 'H-E-L')*, to the gel **[2]** and incubate it overnight in a humidity chamber at 4 degrees Celsius, covered with aluminum foil **[3]**.

3.2.1. *Use 3.1.2.*

3.2.2. Talent adding HEL to a gel. **TEXT: 100 µg/mL HEL** Videographer NOTE: take 2

3.2.3. Talent putting the gel in the humidity chamber.

3.3. After the incubation, remove the HEL antigen and wash the gel with PBS 3 times **[1]**. Finally, cover the gel with 500 microliters of B cell culture media and leave it at room temperature **[2]**.

3.3.1. Talent removing HEL from the gel and adding PBS. Videographer NOTE: take 2

3.3.2. Talent adding media to the gel.

4. Cell Loading and Imaging

4.1. Use a confocal microscope with thermal and carbon dioxide control for imaging **[1]**. Aspirate the media from the gel, leaving approximately 200 microliters **[2]**. Position the gel on the microscope **[3]**. Two main layers of beads will appear on the bottom and the top of the gel. Focus on the gel plane and find an even area to image **[4]**. *Videographer: This step is difficult and important!*

4.1.1. Confocal microscope setup. Videographer NOTE: MED + CU

4.1.2. Talent aspirating the media from the gel.

4.1.3. Talent positioning the gel on the microscope.

4.1.4. SCREEN: Layer of beads coming into focus, then talent looking for and finding the appropriate area for imaging. NOTE: Authors haven't uploaded SC for this and 4.3.2.

4.2. **Judith Pineau:** Pick the imaging area carefully and make sure to stay in focus. An appropriate bead density and an even surface are key to obtaining robust and reliable force measurements.

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

4.3. Add 80 microliters of primary B lymphocytes from MD4 mice to the gel, avoiding touching the gel to maintain focus **[1-TXT]**. Ensure that the focus is still correct and that cells can be seen descending in the area. Launch the acquisition before the cells reach the gel **[2]**.

4.3.1. Talent adding cells to the gel. **TEXT: 3.10^6 cells/mL** Videographer NOTE: cut before the broken glass cover

4.3.2. SCREEN: Cells descending in the gel and acquisition launched.

5. Analysis

5.1. Open the movie as a stack of images in ImageJ, then run the macro "Crop_and_save.ijm". Choose an output directory and configure the attribute channel settings. Select the regions of interest with the **Rectangle** tool and add them to the ROI list using the **t** key. When finished, click **Ok [1]**.

5.1.1. SCREEN: 60947_screenshot_1.mp4. 1:00 – 1:40.

5.2. When the macro proposes a mask of the cell, click **Ok** if it is satisfactory. If not satisfactory, click **Not Ok** and then manually select a closed region with any selection tool, then click **Continue [1]**.

5.2.1. SCREEN: 60947_screenshot_1.mp4. 1:40 – 2:04.

5.3. Open MATLAB and run "TFM_v1.m". Input the required parameters. Specifically, check image properties such as pixel size and time interval of acquisition and the gel properties such as Young modulus E and Poisson ratio. When finished, locate the outputs of the software in the same directory as the original file **[1]**.

5.3.1. SCREEN: 60947_screenshot_1.mp4. 0:02 – 0:30.

Results

6. Results: Information Extracted from Force and Displacement Fields

6.1. Correct bead images look like a uniform and random distribution of bright spots, similar to a starry sky **[1]**. Data and analysis are not reliable when the number of beads is too low **[2]** or the image is out of focus **[3]**.

6.1.1. LAB MEDIA: Figure 2 A.

6.1.2. LAB MEDIA: Figure 2 B and C. *Video Editor: Emphasize B.*

6.1.3. LAB MEDIA: Figure 2 B and C. *Video Editor: Emphasize C.*

6.2. It is possible to observe the movement of beads by eye using a reference frame that preceded the first contact of the cell with the substrate. Approximate results can be obtained from single particle tracking. The analysis provides a segmentation of the beads in the reference image as a control **[1]**.

6.2.1. LAB MEDIA: Figure 3 A.

6.3. Using software, it is also possible to obtain the displacement **[1]** and stress field, which is the vector of the local stress at each pixel and each time point **[2]**. Scalar product of the displacement and force fields integrated on the area of the cell provides total work exerted by the cell on the substrate **[3]**.

6.3.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize B.*

6.3.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize C.*

6.3.3. LAB MEDIA: Figure 4 A.

6.4. When comparing two biological conditions, an average curve **[1]** or an average value over the last time points where the energy reaches a plateau can be calculated **[2]**. When spatial information of the forces is relevant, it is also possible to compare single time points of each condition **[3]**.

6.4.1. LAB MEDIA: Figure 4 B.

6.4.2. LAB MEDIA: Figure 4 C.

6.4.3. LAB MEDIA: Figure 4 D.

6.5. An example of fluorescence antigen extraction time lapse is shown here. The progressive appearance of fluorescence signals at the synapse indicates antigen detachment from the gel **[1]**. The fluorescence data can be used to construct an average extraction curve **[2]**.

6.5.1. LAB MEDIA: Figure 5 A.

6.5.2. LAB MEDIA: Figure 5 B.

Conclusion

7. Conclusion Interview Statements

7.1. **Anita Kumari:** The most delicate step in this protocol is the polymerization of the gel under the coverslip. This step must be performed relatively quickly and carefully, ensuring that the gel is squeezed uniformly under the coverslip.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.8.1 – 2.8.2.*

7.2. **Judith Pineau:** Following this procedure, fluorescent-protein expressing B lymphocytes can be used to simultaneously assess the localization of intracellular structure and force patterning.

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

7.3. **Paolo Pierobon:** This technique can be combined with genetic or chemical perturbations to assess the role of specific proteins on cell contratility and antigen uptake.

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