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Title: Mechanical Mapping of Spheroids Using Brillouin Spectroscopy

Authors and Affiliations:
Chloe B Rodgers[1,*], Giedrė Astrauskaitė[1,*], Rebecca E. Ginesi[1], Greg Wardle[2], Troy R Allen[2], Oana Dobre[1], Massimo Vassalli[1], Matthew Walker[1]

[1]Centre for the Cellular Microenvironment, Advanced Research Centre, University of Glasgow
[2]LightMachinery Inc

*These authors contributed equally

Corresponding Authors:
Matthew Walker matthew.walker@glasgow.ac.uk

Email Addresses for All Authors:
Chloe B Rodgers chloe.rodgers@glasgow.ac.uk
Giedrė Astrauskaitė giedre.astrauskaite@glasgow.ac.uk
Rebecca E. Ginesi rebecca.ginesi@glasgow.ac.uk
Troy R Allen tallen@lightmachinery.com
Greg Wardle gwardle@lightmachinery.com
Oana Dobre oana.dobre@glasgow.ac.uk
Massimo Vassalli Massimo.Vassalli@glasgow.ac.uk
Matthew Walker matthew.walker@glasgow.ac.uk

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**

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

3. **Filming location:** Will the filming need to take place in multiple locations? **YES**

   If **Yes**, how far apart are the locations? Within the same building on different floors; very short distance

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **YES**

Current Protocol Length
Number of Steps: 24
Number of Shots: 56 (27 SC)

Introduction

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

INTRODUCTION:

1.1. **Chloe Rodgers:** We are using Brillouin spectroscopy to non-invasively assess cell mechanics to understand the role of mechanobiology in disease progression.

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

1.2. **Giedrė Astrauskaitė:** Non-invasive technologies, such as Brillouin spectroscopy, allow us to characterize mechanics in three dimensions without disturbing the sample.

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

CONCLUSION:

1.3. **Matthew Walker:** Our protocol will provide a platform that will enable researchers to study many diseases linked to mechanobiology.

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

1.4. **Chloe Rodgers:** It will be interesting to see if changes in Brillouin shift that correlate with cell mechanical properties are effective markers for the stages of disease progression.

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

1.5. **Giedrė Astrauskaitė:** We will assess how drug treatments influence spheroid mechanics over timescales that mimic disease progression.

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

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

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

1.6. **Dr. Matthew J Walker, Senior Researcher** (authors will present their testimonial statements live)

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Dr. Matthew J Walker, Senior Researcher**: (authors will present their testimonial statements live)

Protocol

2. Preparation of Spheroids

Demonstrator: Chloe Rodgers

2.1. To begin, pre-treat the wells of the microwell plate by adding 500 microliters of anti-adherence rinsing solution to each well **[1]**. Centrifuge the plate at 1,300 *g* for 5 minutes at room temperature **[2]**. After eliminating air bubbles, remove the anti-adherence rinsing solution from the wells **[3]**.

2.1.1. **WIDE:** Talent adding 500 microliters of anti-adherence rinsing solution into each well of the microwell plate using a pipette.

2.1.2. Talent placing the plate inside the centrifuge and setting it to 1,300 g for 5 minutes at room temperature.

2.1.3. Talent aspirating and removing the anti-adherence rinsing solution from the wells.

2.2. Next, add 1 milliliter of PBS to each well and aspirate it completely **[1]**. After repeating the PBS wash, determine the number of cells per spheroid and the number of spheroids per gel according to the experimental design **[2-TXT]**.

2.2.1. Talent dispensing 1 milliliter of PBS into each well and aspirating it out.

2.2.2. Talent recording or noting the calculated cell number in a notebook. **TXT: Calculate the number of cells required to obtain 1.2 × 10^5 cells/well**

2.3. Pellet the cells and resuspend them in 1 milliliter of supplemented DMEM **[1].** Then add 1 milliliter of this cell suspension into one well of the microwell plate and mix well **[2]**. Centrifuge the plate at 100 *g* for 3 minutes at room temperature **[3]**.

2.3.1. Talent resuspending the cell pellet in 1 milliliter of the described culture medium.

2.3.2. Talent adding 1 milliliter of the cell suspension into a microwell plate well.

2.3.3. Talent placing the plate into the centrifuge.

2.4. Observe the microwell plate under the microscope to confirm even distribution of cells and that they have settled at the bottom of the wells **[1]**. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide for 24 hours to allow spheroid formation

[2].

2.4.1. Talent placing the plate under a microscope.

2.4.2. Talent placing the plate into an incubator set to 37 degrees Celsius and 5 percent carbon dioxide.

2.5. To harvest the spheroids, gently and slowly remove the culture media without disturbing the spheroids **[1]**. Cut the PBS-FBS-coated pipette tip to widen the opening **[2]** and add 500 microliters of PBS to each well to dislodge the spheroids **[3]**. Collect the PBS containing the spheroids into a tube **[4]**.

2.5.1. Talent aspirating and removing the culture media carefully from the microwell plate.

2.5.2. Talent cutting the pipette tip to create a wider opening.

2.5.3. Talent adding 500 microliters of PBS to each well and pipetting up and down to dislodge spheroids.

2.5.4. Talent transferring the PBS containing spheroids into a collection tube.

2.6. Then, transfer 50 microliters of the spheroid suspension into one well of a 96-well plate **[1]**. To aid counting, draw a cross on the bottom of the well using a permanent marker **[2]** and count the number of spheroids in this volume under the microscope **[3]**. Use the equation to calculate the total number of spheroids: **[4]**

2.6.1. Talent transferring 50 microliters of spheroid suspension into a 96-well plate.

2.6.2. Talent drawing a cross under the well with a permanent marker to assist with counting.

2.6.3. Talent viewing the sample under a microscope.

2.6.4. TEXT ON PLAIN BACKGROUND:

$$No.of\ spheroids = \frac{Volume\ of\ spheroid\ suspension\ (\mu L)}{50\ \mu L} \times Spheroid\ count\ in\ 50\ \mu L$$

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3. Setting up the Brillouin Microscope

Demonstrator: Giedrė Astrauskaitė

3.1. Open the **SpectraLok** software and wait until all connected devices are detected **[1-TXT]**. Then, select the spectrometer camera **[2]**.

3.1.1. SCREEN: Shot-3.1.1+3.1.2+3.10.1.mp4, 00:00-00:05. **TXT: Turn on and check all**

the devices beforehand

3.1.2. SCREEN: Shot-3.1.1+3.1.2+3.10.1.mp4, 00:05-00:10.

3.2. Install the acrylic cube on the microscope sample holder with the clear side facing the objective lens **[1]**. Lower the objective lens as much as possible and center the cube beneath it **[2]**. Ensure that the optical path in the microscope is set so that the spectrometer camera is exposed by turning the rotary port selector to L **[3]**.

3.2.1. Talent placing the acrylic cube onto the microscope stage, clear side facing the objective lens.

3.2.2. Close-up shot of the objective lens being lowered and aligned over the cube.

3.2.3. Talent adjusting the rotary port selector on the microscope to the **L** position.

3.3. In the **SpectraLok Camera** window, set the sensor exposure time to 500 milliseconds and the software gain to 100 **[1-TXT]**.

3.3.1. SCREEN: Shot-3.3.1.mp4. **TXT: Unblock the laser shutter**

3.4. Press **Capture** and slowly move the objective lens upward **[1]**. Continue raising the lens until the Brillouin peaks become visible and their intensity stabilizes, indicating that the focal spot is positioned within the acrylic cube **[2]**.

3.4.1. SCREEN: Shot-3.4.1.mp4.

3.4.2. SCREEN: Shot-3.4.2.mp4. 00:07-00:20

3.5. Now, open the **Pump Killer or PK** control window **[1]**.

3.5.1. SCREEN: Shot-3.5.1.mp4. 00:03-00:08

3.6. Zoom in on one of the brightest Brillouin orders and adjust the pressure actuator position in increments of 100 micrometers by clicking **Move Rel** to minimize the Rayleigh peak intensity **[1]**. If the Rayleigh signal increases, click **Reverse** to change the adjustment direction **[2]**.

3.6.1. SCREEN: Shot-3.6.1+3.6.2.mp4 00:05-00:20.

3.6.2. SCREEN: Shot-3.6.1+3.6.2.mp4 00:20-00:25.

3.7. Once the Rayleigh peak intensity decreases, fine-tune the etalon pressure in smaller increments of 10 micrometers **[1]**. In the camera window, zoom in on one of the

Brillouin orders **[2]**.

3.7.1. SCREEN: Shot-3.7.1.mp4 00:05-00:20

3.7.2. SCREEN: Shot-3.7.2.mp4. 00:09-00:14

3.8. Press **S** to visualize stripes across which the LUT is applied to the camera image **[1]**. Open the **Settings** window and press **Quick Calibrate** to automatically recalculate the optimal horizontal offset of the stripes **[2-TXT]**.

3.8.1. SCREEN: Shot-3.8.1.mp4 00:05-00:15.

3.8.2. SCREEN: Shot-3.8.1.mp4 00:03-00:15. **TXT: If the sensor becomes saturated, reduce the exposure time**

3.9. After verifying the stripe-alignment, adjust the collimator lens axes to maximize the coupling efficiency of light from the **Pump Killer** to the spectrometer **[1]**. Then, open the **Spectrum** window and click **Unwrap** to retrieve the spectrum **[2-TXT]**.

3.9.1. SCREEN: Shot-3.9.1+3.9.2.mp4. 00:25-00:37.

3.9.2. SCREEN: Shot-3.9.1+3.9.2.mp4. 00:00-00:10. **TXT: Adjust the collimator axes 1 and 2 in increments of 0.0005°**

3.10. In a homogeneous acrylic cube, aim for complete suppression of the Rayleigh signal and adjust until the Brillouin signal is clearly visible **[1-TXT]**.

3.10.1. SCREEN: Shot-3.10.2.mp4 00:05-00:23. **TXT: Brillouin signal should be comparable to reference amplitude**

3.11. To save the optimized parameters, click **Refresh All** and then **Save All** in the **PK Control** window **[1]**. Finally, in the main window, click **Save** in the **File I/O** *(I-O)* section to store the configuration **[2]**.

3.11.1. SCREEN: Shot-3.11.1.mp4 00:08-00:21.

3.11.2. SCREEN: Shot-3.11.2.mp4 00:05-00:17.

4. Mounting the Sample and Image Acquisition

4.1. After opening the scanning software, install the glass-bottom petri dish containing the hydrogel-spheroid sample in place of the acrylic cube **[1-TXT]**.

4.1.1. Talent placing the glass-bottom petri dish with the hydrogel-spheroid sample onto the microscope stage. **TXT: Incubator settings: 37 °C and 95% relative humidity**

4.2. Remove the petri dish lid, add sufficient media to submerge the gel **[1]**, and place a coverslip on top to prevent gel movement **[2]**.

4.2.1. Talent removing the petri dish lid and adding media to fully submerge the gel.

4.2.2. Close-up shot of the coverslip being placed gently over the gel to prevent movement.

4.3. Locate the spheroid by first blocking the laser beam **[1]**. Switch the microscope optical path to the eyepiece and turn on the white light illumination **[2]**. Using the joystick, manually control the sample stage to locate and focus on the spheroid **[3]**.

4.3.1. Talent rotating the wheel to block the laser beam.

4.3.2. Talent switching the optical path selector to the eyepiece and turning on white light illumination.

4.3.3. Talent using the joystick to move the stage and bring the spheroid into focus through the eyepiece.

4.4. Acquire a brightfield image of the spheroid and adjust the illumination brightness by rotating the control wheel **[1]**. Switch the optical path to **R** to expose the microscope camera and set the exposure time as required **[2]**.

4.4.1. SCREEN: Shot-4.1.1.mp4 00:08-00:20.

4.4.2. Talent rotating the optical path selector to **R** for camera exposure.

4.4.3. ~~SCREEN: Display exposure time adjustment to 50 milliseconds to enhance image contrast~~.

4.5. Next, set the microscope port to **L** and capture the background with the laser still blocked **[1]**. Unblock the laser and depending on the desired signal-to-noise ratio, select an exposure time between 200 and 500 milliseconds **[2-TXT]**.

4.5.1. Talent setting the port selector to **L**.

4.5.2. SCREEN: Shot-4.5.2.mp4 00:19-00:30. **TXT: Re-optimize etalon pressure and pump killer-spectrometer coupling if disturbed**

4.6. From the unwrapped spectrum, select the Brillouin peak fitting range **[1]**. Adjust the threshold value to approximately half the Brillouin peak intensity **[2]** and exclude any Brillouin peaks with amplitude below the threshold **[3]**. Apply wavelength correction to the laser according to all identified Brillouin peak pairs **[4]**.

4.6.1. SCREEN: Shot-4.6.mp4 00:10-00:20.

4.6.2. SCREEN: Shot-4.6.mp4 00:20-00:30.

4.6.3. SCREEN: Shot-4.6.mp4 00:32-00:42.

4.6.4. SCREEN: Shot-4.6.mp4 00:50-01:05.

4.7. Finally, choose the raster scan geometry by adjusting the scan width and step size **[1]** and start the scan to record the measurements **[2]**.

4.7.1. SCREEN: Shot-4.7.mp4 00:05-00:20.

4.7.2. SCREEN: Shot-4.7.mp4 00:21-00:28.

Results

5. Results

5.1. Representative images of a mesenchymal stem cell spheroid embedded in hydrogel showed distinct Brillouin Shift **[1]** and Brillouin Linewidth **[2]** maps, along with corresponding brightfield **[3]**, nuclei **[4]**, and actin filament fluorescence images **[5]**.

5.1.1. LAB MEDIA: Figure 9A.

5.1.2. LAB MEDIA: Figure 9B.

5.1.3. LAB MEDIA: Figure 9C.

5.1.4. LAB MEDIA: Figure 9D.

5.1.5. LAB MEDIA: Figure 9E.

5.2. The low-resolution Brillouin Frequency Shift map was segmented into gel and spheroid regions **[1]** based on the contour manually traced from the brightfield image **[2]**.

5.2.1. LAB MEDIA: Figure 10A.

5.2.2. LAB MEDIA: Figure 10B.

5.3. Ambiguous pixels resulting from down-sampling were excluded from analysis **[1]**, while clear binary masks for the gel **[2]** and spheroid regions were created to extract BFS values **[3]**.

5.3.1. LAB MEDIA: Figure 10B. *Video editor: Highlight the "Excluded values" panel*

5.3.2. LAB MEDIA: Figure 10C.

5.3.3. LAB MEDIA: Figure 10D.

5.4. Comparison of high- and low-resolution BFS maps demonstrated that coarse sampling provided mean BFS values comparable to high-resolution scans, indicating consistent measurement of spheroid bulk mechanics **[1]**.

5.4.1. LAB MEDIA: Figure 11. *Video editor: Highlight the corresponding BFS values "6.48….." in the bottom row's three images*

- **centrifuge**

Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge Merriam-Webster

IPA: /ˈsɛn.trə.fjuʤ/
Phonetic Spelling: sen-truh-fyooj

- **microwell**
Pronunciation link: No confirmed link found
IPA (approximation): /ˈmaɪ.kroʊ.wɛl/
Phonetic Spelling: my-kroh-wel

- **anti-adherence**
Pronunciation link: No confirmed link found
IPA (approximation): /ˌæn.taɪ əˈdɪr.əns/
Phonetic Spelling: an-ty-uh-di-ruhnss

- **spheroid**
Pronunciation link: No confirmed link found
IPA (approximation): /ˈsfɪə.rɔɪd/
Phonetic Spelling: sfee-uh-roid

- **spectrometer**
Pronunciation link: No confirmed link found
IPA (approximation): /spɛkˈtrɑː.mə.tər/
Phonetic Spelling: spek-trow-meh-ter

- **etalon** *(optics term)*
Pronunciation link: No confirmed link found
IPA (approximation): /ˈeɪ.tæ.lɑn/
Phonetic Spelling: ay-ta-lon

- **collimator**
Pronunciation link: No confirmed link found
IPA (approximation): /kəˈlɪm.ə.tər/
Phonetic Spelling: kuh-lim-uh-ter

- **fluorescence**
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence Merriam-Webster+1
IPA: /flɔrˈɛsəns/
Phonetic Spelling: flor-es-uhns

- **segmented**
Pronunciation link: No confirmed link found (common word)
IPA (approximation): /ˈsɛg.mən.tɪd/
Phonetic Spelling: seg-muhn-tid

- **down-sampling**

Pronunciation link: No confirmed link found (compound)
IPA (approximation): /ˈdaʊnˌsæm.pəlɪŋ/
Phonetic Spelling: down-sam-puh-ling