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Title: Near Simultaneous Laser Scanning Confocal and Atomic Force Microscopy (Conpokal) on Live Cells

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Author Questionnaire

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

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

Y

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

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonably possible.

Videographer: Screen captures not provided, please film screen for backup

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

Protocol Length
Number of Shots: **44**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Martha Grady**: Conpokal combines confocal microscopy with atomic force microscopy, using a probe to "poke" the sample surface. Although both techniques are effective individually, Conpokal facilitates fluorescence co-localization with mechanical characterization **[1]**.

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

REQUIRED:

1.2. **Joree Sandin**: The main advantage of the Conpokal technique is the near-simultaneous dual microscopy. Confocal investigates cytoskeletal and other cellular processes before and after AFM probing, which delivers area-specific mechanical properties **[1]**.

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

OPTIONAL:

1.3. **Surya Aryal**: This technique is impactful within the mechanobiology field as, for example, brain cells can be probed under physiological conditions to examine electrical impulses and force transduction **[1]**.

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

Protocol

2. Atomic Force Microscopy (AFM)

2.1. Before beginning the analysis, select an appropriate AFM (A-F-M) cantilever for the desired data collection **[1]** and use gloves, the AFM chip mounting stage, tweezers, and a small screwdriver to mount the tip into the cantilever glass block **[2]**.

2.1.1. WIDE: Talent selecting cantilever

2.1.2. Talent mounting glass block into mounting stage

2.2. Carefully place the AFM chip onto the center of the glass block **[1]**. The cantilever, plus a very small portion of the AFM chip, should be in the visible, non-opaque portion of the glass block **[2]**.

2.2.1. Chip being placed into center of glass block *Videographer: Important/difficult step*

2.2.2. Shot of cantilever plus chip in non-opaque portion of glass block *Videographer: Important/difficult step*

2.3. Use the screwdriver to tighten the screw until the tip is snug against the glass block **[1]** and use a lens to check that the tip is oriented correctly **[2-TXT]**.

2.3.1. Screw being tightened *Videographer: Important step*

2.3.2. Talent checking orientation *Videographer: Important step* **TEXT: *e.g.*, use stereomicroscope or handheld spyglass** Author NOTE: A stereo microscope was used to check the orientation. The videographer collected a wide view shot of this and a microscope-camera shot as well.

2.4. When the tip has been properly oriented, place the glass block into the AFM head in the proper orientation **[1]** and lock the glass block into place **[2]**.

2.4.1. Talent placing glass block into AFM head

2.4.2. Talent locking glass block into place

2.5. After locating the bottom of the calibration dish by brightfield confocal microscopy **[1]**, use the AFM system z stepper motor control panel to move the AFM cantilever 2000 microns above the sample **[2]**.

2.5.1. Talent at microscope, locating dish, with monitor visible in frame
2.5.2. SCREEN: JoVE_AFMcomputer_2.5.2.mp4. Cantilever being moved

~~2.6. **[1]**.~~

~~2.6.1. SCREEN: To be provided by Authors: Chip being lowered, then manual micrometers being located~~

~~2.7. **[1]**.~~

~~2.7.1. SCREEN: To be provided by Authors: Head being corrected, then position being adjusted~~

2.8. Using brightfield illumination and the z-stepper motor control panel, slowly lower the AFM chip to the bottom of the glass dish in steps of 100 to 200 microns to avoid crashing the AFM tip into the Petri dish. Watch for a shadow to appear in the microscope software view, which indicates that the AFM tip is getting closer to the bottom of the dish **[1]**.

2.8.1. SCREEN: Chip being lowered (should be in video JoVE_AFMcomputer_2.8.2.mp4: Cantilever being lowered/shadow appearing

2.9. Locate the manual micrometers that control x-y or in-plane motion of the AFM head on the insturment platform **[1]**. As the shadow from the AFM cantilever becomes darker and the shape becomes sharper, use the manual micrometers to correct the AFM head and adjust the postion of the AFM cantilever within the field of view. At this time, the look up tables may need to be adjusted **[2]**.

2.9.1. WIDE: Manual micrometers being located and adjusted. NOTE: This slating may not match the videographer's footage. Maybe 2.8.1 here: Cantilever being lowered
2.9.2. SCREEN: Tip being moved to the center of the field of view (should be in video named JoVE_Confocalcomputer_2.8.2.mp4)

2.10. Continue using small steps to lower the AFM tip until the tip is mostly in-focus **[1]**.

2.10.1. SCREEN: JoVE_AFMcomputer_2.10.1.mp4. and JoVE_Confocalcomputer_2.9.1and2.10.1and2.11.1.mp4: Tip being lowered

2.11. When the tip is dark enough that its general shape can be observed centered in the field of view while still remaining blurry, adjust the microscope focus so the tip becomes clear **[1]** and remove the laser light filter **[2]**.

2.11.1. SCREEN: JoVE_Confocalcomputer_2.9.1and2.10.1and2.11.1.mp4. Shot of dark, blurry tip, then tip coming into focus
2.11.2. Talent removing laser light filter

2.12. With the laser light visible in the optics, use the laser alignment dials to move the laser into the field of view near the AFM tip **[1]**.

2.12.1. SCREEN: JoVE_Confocalcomputer_2.12.1and2.13.2.mp4. Laser being moved into FOV

2.13. When the laser is in position, **[1]** use the laser alignment dials to position the laser on the backside of where the AFM tip is located on the cantilever **[2]**.

2.13.1. Talent replacing laser light filter NOTE: Author thinks this should be moved to 2.18.1.
2.13.2. SCREEN: JoVE_Confocalcomputer_2.12.1and2.13.2.mp4laser being positioned

2.14. The laser alignment panel should display a sum signal greater than 0 volts **[1-TXT]**.

2.14.1. SCREEN: JoVE_AFMcomputer_2.14.1.mp4. Shot of sum signal Video Editor: please emphasize sum signal when mentioned **TEXT: If no signal, manually adjust controlled mirror knob until >0 V sum signal obtained**

2.15. Move the laser a small distance in all of the directions on the AFM cantilever until the maximum sum signal is achieved while remaining at the AFM tip. Once the laser position is set, use the manually controlled deflection dials to zero the vertical and lateral deflection **[1]**. NOTE: The last sentence is the same action as 2.16.1.

2.15.1. SCREEN: JoVE_Confocalcomputer_2.15.1.mp4 and JoVE_AFMcomputer_2.15.1.mp4. Laser being moved, then vertical and lateral deflections being zeroed

2.16. Use the vertical and lateral deflection knobs to align the detector so that the target is centered and there is no vertical or lateral deflection observed in the laser alignment panel **[1]**.

2.16.1. Aligning the detector NOTE: This was supposed to be screen but the authors may have changed it to videographer's footage. If no footage is available show the previous SC 2.15.1.

2.17. Open the calibration window and enter all of the experiment specific information **[1]**.

2.17.1. SCREEN: JoVE_AFMcomputer_2.17.1.mp4. Window being opened and information being entered

2.18. Replace the laser light filter. Before calibrating, turn off the confocal microscope light source **[1]** and close the AFM enclosure to dampen any potential noise coming from the room light or vibrations **[2]**.

2.18.1. SCREEN: JoVE_Confocalcomputer_2.18.1.mp4. Light source being turned off
2.18.2. Talent closing enclosure

2.19. Press the calibration button to automatically allow the system to calibrate the tip **[1]**.

2.19.1. SCREEN: JoVE_AFMcomputer_2.19.1and2.20.1.mp4: Pressing calibration button

2.20. When the calibration is complete, the stiffness of the cantilever and its sensitivity will be displayed in the calibration panel **[1-TXT]**.

2.20.1. SCREEN: JoVE_AFMcomputer_2.19.1and2.20.1.mp4: Shot of cantilever stiffness and sensitivity data *Video Editor: please emphasize stiffness and sensitivity when mentioned* **TEXT: Note data for later analysis**

2.21. Then use the automated approach command button to lower the tip to the bottom of the sample dish and set the size of the scan area, the resolution, the setpoint, the z length, and the pixel time before pressing the "Play" button to begin scanning **[1]**.

2.21.1. SCREEN: JoVE_AFMcomputer_2.21.1.mp4 and JoVE_AFMcomputer_2.21.2.mp4: Tip being lowered, scanning parameters being set, then scanning being initiated Author NOTE: The ...2.21.2 file was additional to show what happens with the scan finishes, showing that the file gets saved

3. Confocal Microscopy

3.1. For confocal microscope imaging, in the microscope software, enable the confocal capabilities **[1-TXT]** and select the laser lines appropriate for the dyes that were used to stain the samples **[2]**.

3.1.1. WIDE: Talent enabling capabilities, with monitor visible in frame **TEXT: Confocal microscopy option typically noted by laser line wavelength values**
3.1.2. SCREEN: JoVE_Confocalcomputer_3.1.2.mp4: Laser lines being selected

3.2. Turn on one or multiple laser lines to excite and image those features in the sample and set the gain to a value that optimizes the sample fluorescence but limits the amount of noise **[1]**.

3.2.1. SCREEN: JoVE_Confocalcomputer_3.2.1.mp4: Laser line being turned on, then gain being set

3.3. Adjust the laser power to avoid saturated pixels while maximizing the dynamic range and set the pinhole size to 1 Airy unit to maximize the resolution for the optical sectioning **[1-TXT]**.

3.3.1. SCREEN: JoVE_Confocalcomputer_3.3.1.mp4: Laser power being adjusted, then pinhole size being set **TEXT: If dim sample with high laser power, opening pinhole increases signal but decreases z-axis resolution**

3.4. To set the pixel dwell time, begin with a 2-microsecond dwell time and adjust to reflect the sample brightness as needed **[1]**.

3.4.1. SCREEN: JoVE_Confocalcomputer_3.4.1.mp4: Dwell time being set to 2 ms, then being adjusted

3.5. To select the pixel size for the selected objective, let the instrument calculate the pixel size via the Nyquist option button and the selected number of pixels in the image **[1]**.

3.5.1. SCREEN: JoVE_Confocalcomputer_3.5.1.mp4: Instrument calculating pixel size

3.6. Next, select the **Scan** option and begin the data collection **[1]**.

3.6.1. SCREEN: JoVE_Confocalcomputer_3.6.1.mp4: Scan being selected, then collection being initiated

3.7. Use the focus knob to zoom in and out to locate the optimal field of view in the sample and use the **Capture** button to capture images **[1-TXT]**. Using the panel which controls the pixel size via the Nyquist option, reduce the area size of the scan to only envelop a single cell **[added]**.

3.7.1. SCREEN: oVE_Confocalcomputer_3.7.1.mp4: FOV being identified, then Capture being clicked **TEXT: See in appropriate format and location** NOTE: LUTs is adjusted

3.7.2. Added: SCREEN: JoVE_Confocalcomputer_btw3.7.1and3.8.1.mp4 to show users how to isolate a single cell in the sample.

3.8. Activate the collection tool and, using a bottom-to-top option with only the laser line that illuminates a feature in the sample most clearly, set the start and finish planes for the volume to be measured, using the suggested spacing between the planes. Name the file and save it to the associated folder **[1-TXT]**.

3.8.1. SCREEN: JoVE_Confocalcomputer_3.8.1.mp4: Tool being activated and start and finish planes being set **TEXT: Spacing calculated to fulfill Nyquist sampling criteria**

3.9. If a priori knowledge of the sample's thickness exists, select the brightest, sharpest middle plane to define the volume and set the top to half the sample thickness above the middle plane and the bottom to half the thickness below the middle plane **[1]**.

3.9.1. SCREEN: JoVE_Confocalcomputer_3.9.1.mp4: Plane being selected, then thickness being set

3.10. Run the acquisition by pressing the "**Run Now**" button **[1]**. When the acquisition finishes, save the file to the appropriate folder **[2-added]**.

3.10.1. SCREEN: JoVE_Confocalcomputer_3.10.1.mp4
Added: SCREEN: JoVE_Confocalcomputer_3.10.2.mp4: File being saved Author NOTE: 3.10.2 to show the scan finishing and the file being saved

4. Clean Up

4.1. At the end of the analysis, wear gloves while removing the AFM chip holder **[1]** and place the holder into the mounting station **[2]**.

4.1.1. WIDE: Talent wearing gloves removing holder
4.1.2. Talent placing holder into mounting station

4.2. Use tweezers with rubber grips to carefully remove the AFM chip **[1]** and place the used tip back into the location of the storage box oriented off-axis to denote that it has been used **[2]**.

4.2.1. Chip being removed
4.2.2. Chip being placed into box

4.3. For future reference, note which tip was used in the experiment **[1]**.

4.3.1. Talent looking at box, noting chip in notebook

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.2., 2.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.2., 2.8.

Results

5. Results: Representative Conpokal Live Cell Imaging

5.1. Here a representative AFM scan over a living HEK (H-E-K) cell is shown **[1-TXT]**.

5.1.1. LAB MEDIA: Figures 2A and 2B
5.1.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize Figure 2A* **TEXT: HEK: human embryonic kidney**

5.2. The height for this particular HEK cell was around 10 microns **[1]**, as demonstrated by the line scan **[2]**.

5.2.1. LAB MEDIA: Figures 2A and 2B
5.2.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize green line in Figure 2B*

5.3. Here an example of a poor scan due to an improper AFM tip choice can be observed **[1]**.

5.3.1. LAB MEDIA: Figure 2C

5.4. In this image, black pixels appeared at the apex of the cell **[1]**, indicating that the AFM piezo was out of range due to a large cell height **[2]**.

5.4.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize black pixels in center of image*
5.4.2. LAB MEDIA: Figure 2C

5.5. The end of the AFM cantilever also appears in the image because of the tip offset combined with an insufficient tip height compared to the cell height **[1]**.

5.5.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize rounded square at bottom of image*

5.6. These artifacts in the AFM image indicate that a different AFM tip should have been chosen to image the cell **[1]**.

5.6.1. LAB MEDIA: Figure 2C

5.7. Three-color confocal imaging can be performed **[1]**, for example to visualize the cell nucleus **[2]**, microtubules **[3]**, and lipophilic membrane **[4]**.

5.7.1. LAB MEDIA: Figure 3A
5.7.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize blue signal*
5.7.3. LAB MEDIA: Figure 3A *Video Editor: please emphasize green signal*
5.7.4. LAB MEDIA: Figure 3A *Video Editor: please emphasize red signal*

5.8. Analysis of the tip indentations in combination with a nanomechanical model allows the generation of a modulus map of the surface **[1]**.

5.8.1. LAB MEDIA: Figure 4A

5.9. Here a corresponding 3D projection of the laser confocal z-stack is shown **[1]**.

5.9.1. LAB MEDIA: Figure 4A *Video Editor: please emphasize Figure 4B*

5.10. AFM scans and measured modulus maps can also be acquired for microbes, as observed in these representative analyses of a *Streptococcus mutans* bacterium **[1]**.

5.10.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize sequentially emphasize Figures 5A and 5B*

5.11. As demonstrated, a better resolution can be achieved at this scale with AFM than with traditional confocal microscopy **[2]**.

5.11.1. LAB MEDIA: Figure 6

Conclusion

6. Conclusion Interview Statements

6.1. **Joree Sandin**: Adhesive force mapping is a technique performed through Conpokal to visualize molecular interactions, for example, to study the modulation of cell surface molecules to observe binding kinetics **[1]**.

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

6.2. **Martha Grady**: Conpokal ushers in a new pathway for exploring structure-function relationships in medical microbiology. For example, physical and chemical events in the peptidoglycan layer can be linked to antibiotic resistance **[1]**.

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