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Title: Silicon Nanowires and Optical Stimulation for Investigations of Intra- and Intercellular Electrical Coupling

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate 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**

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

Protocol Length
Number of Shots: **42**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Menahem Y. Rotenberg**: This technique allows intracellular bioelectrical interrogation with an extremely high spatial resolution and cell specificity using standard optical microscopy **[1]**.

1.1.1. LAB MEDIA: To be provided by Authors by 19 Sep: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Menahem Y. Rotenberg**: This method facilitates the stimulation and electrical interrogation of specific cell types and locations within cells and can be performed in in vitro and 3D ex vivo tissue preparations **[1]**.

1.2.1. LAB MEDIA: To be provided by Authors by 19 Sep: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Aleksander Prominski**: This technique enables the bioelectric investigation of many cellular arenas, such as cardiac or brain cells, as well the electrical communication between other non-excitable cells **[1]**.

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

Protocol

2. Cell-Silicon Nanowire (SiNWs) Hybrid Preparation

2.1. To isolate myofibroblasts from a myofibroblast-cardiomyocyte suspension, pre-plate the isolated cells on a 100-millimeter tissue culture dish for 1 hour **[1]**.

2.1.1. WIDE: Talent adding cells to plate

2.2. As cardiomyocytes need a fibronectin-treated surface to adhere, at the end of the incubation, collect the enriched cardiomyocyte-containing supernatant for downstream co-culture **[1]**

2.2.1. Talent adding supernatant to collection container

2.3. Rinse the myofibroblasts with DMEM (D-M-E-M) to eliminate any remaining cardiomyocytes **[1-TXT]** and feed the cells with fresh culture medium before an additional 2-4 days of incubation **[2-TXT]**.

2.3.1. Talent rinsing dish, with medium container visible in frame **TEXT: DMEM: Dulbecco's modified Eagle medium**

2.3.2. Talent adding medium to dish **TEXT: Change medium every other day**

2.4. When the cells reach 80% confluency, use a diamond scribe to cut a 3- x 3-millimeter chip from a wafer with chemical vapor deposition-grown silicon nanowires **[1]** and use sharp forceps to rinse the chips in 70% ethanol **[2]**.

2.4.1. Chip being cut with scribe *Videographer: Important step*

2.4.2. Chip(s) being rinsed *Videographer: Important step*

2.5. After rinsing, air dry the chips for 30 minutes under ultraviolet light in a biosafety laminar flow hood **[1]** before transferring the chips to a sterile microcentrifuge tube **[2]**.

2.5.1. Talent placing chip into hood with other chips, then turning on UV light *Videographer: Important step*

Shot split into 2.5.1A and 2.5.1B:
2.5.1A. Talent placing chip into hood with other chips on a petri dish
2.5.1B. Talent closing the hood and turning on the UV light.

2.5.2. Talent adding chip(s) to tube *Videographer: Important step*

2.6. Use a complete cell culture medium rinse to remove any remaining ethanol **[1-TXT]** before sonicating the chips in 1 milliliter of fresh culture medium in a sonication bath for 1-10 minutes **[2]**. The supernatant should turn cloudy as the nanowires are released **[3]**.

2.6.1. Talent rinsing chips, with medium container visible in frame *Videographer: Important step* **TEXT: See text for all medium and solution preparation details**

2.6.2. Tube being placed in the ultrasonic bath, bath being started *Videographer: Important step*

2.6.3. Tube being taken out of the bath, cloudy solution visible in the shot.

2.7. Mix the silicon nanowire suspension into 5 milliliters of fresh culture medium **[1]** and seed the nanowire solution onto the dish of myofibroblasts **[2]**.

2.7.1. Talent adding SiNWs to tube, with medium container visible in frame *Videographer: Important step*

2.7.2. Talent adding SiNWs to plate *Videographer: Important step*

2.8. After 4 hours in the cell culture incubator, rinse the dish five times with fresh culture medium to remove any uninternalized nanowires **[1]** and continue to incubate the cells for another hour to allow any partially internalized nanowires to be completely incorporated **[2]**.

2.8.1. Talent rinsing dish, with medium container visible in frame

2.8.2. Talent placing dish in incubator and setting timer

2.9. Next, add 500 microliters of freshly prepared collagen coating solution to a 35-millimeter glass bottom dish **[1]**.

2.9.1. Talent adding solution to dish, with collagen and acetic acid containers visible in frame

2.10. After a 1-hour incubation at 37 degrees Celsius, rinse the dish with sterile PBS **[1]** and harvest the silicon nanowire-myofibroblast hybrids with 3 milliliters of trypsin for 2 minutes at 37 degrees Celsius **[2]**.

2.10.1. Talent rinsing dish, with PBS container visible in frame

2.10.2. Talent adding trypsin to dish, with trypsin container visible in frame

2.11. When the cells have detached, stop the reaction with 10 milliliters of culture medium, rinse vigorously by pipetting **[1]**, and collect the cells by centrifugation **[2-TXT]**.

2.11.1. Talent adding medium to dish, washing vigorously, and transferring into the centrifuge tube Video Editor: Please use a shot in which no bubbles are formed during pipetting

2.11.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 200 x g, RT**

2.12. Then resuspend the hybrid cell pellets in 1 milliliter of fresh medium **[1]** and seed the cells on the collagen-coated glass bottom dish **[2]**.

2.12.1. Shot of pellet if visible, then medium being added, with medium container visible in frame
Split into 2 shots:
2.12.1A – shot of a pellet with supernatant above
2.12.1B – shot of a pellet with supernatant already removed, medium being added.
Video Editor: Can remove Shot 1A if prolongs video too much

2.12.2. Talent adding cells to dish

2.13. To verify the nanowire internalization, label the cells with fluorescent cytosol and membrane dyes according to standard protocols **[1-TXT]** and use a microscope to image the cells **[2]**.

2.13.1. Talent adding dye to dish, with dye containers visible in frame **TEXT: For optimal performance, work with dyes under red light**

2.13.2. Talent at microscope, imaging cells

2.14. As silicon nanowires are highly reflective, reflected light can be used instead of fluorescence for their visualization **[1]**.

2.14.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize white signal in right images*

3. Cardiomyocyte (CM):Myofibroblast SiNW Hybrid Co-Culture

3.1. To set up a myofibroblast silicon nanowire hybrid-cardiomyocyte co-culture, seed the appropriate number of each cell type onto a collage-coated glass bottom dish **[1-TXT]** and culture the cells at 37 degrees Celsius for 48 hours to allow the cells to form intercellular gap junctions **[2]**.

3.1.1. WIDE: Talent adding cells to dish, with medium and cell containers visible in frame **TEXT: See text for suggested inter- and intracellular cell density details**

3.1.2. Talent placing dish into incubator

3.2. On the day of the experiment, replace the culture supernatant with 1 milliliter of culture medium supplemented with freshly prepared calcium sensitive dye for a 20-30-minute incubation at 37 degrees Celsius **[1]**.

3.2.1. Talent adding dye solution to dish, with dye container visible in frame

3.3. At the end of the incubation, wash the plate two times with sterile PBS **[1]** before treating the cells with 1 milliliter of pre-warmed, phenol red-free DMEM **[2]**.

3.3.1. Talent washing dish, with PBS container visible in frame

3.3.2. Talent adding medium to dish, with medium container visible in frame

3.4. Then allow the intracellular calcium dye to undergo de-esterification for 30 minutes at 37 degrees Celsius before acquiring baseline images **[1]**.

3.4.1. Talent placing dish into incubator and starting a timer

4. Optical Imaging and Stimulation

4.1. Before optical imaging and stimulation, pre-heat a humidified microincubator on a microscope with a collimated laser line coupled into the light path for calcium imaging **[1]** and optical stimulation to 37-degree Celsius and a 5% bubble air-carbon dioxide mixture **[2]**.

4.1.1. 4.1.1A. Talent turning on microincubator controller, temperature sensors increasing
4.1.1B. Talent opening microincubator to confirm carbon dioxide bubbling

4.2. When the microscope is ready, place the myofibroblast hybrid-cardiomyocyte co-culture into the microincubator **[1]** and visualize the nanowires by brightfield microscopy to locate an appropriate stimulation site **[2]**.

4.2.1. Talent placing cells into microincubator *Videographer: Important step*

4.2.2. Talent at microscope, locating SiNW, with monitor visible in frame *Videographer: Important step* Video Editor: Sample not representative and monitor details should not be clear

4.3. When a site has been identified, reconfigure the light path to fluorescence mode while maintaining the stimulation point at the predefined location of the nanowire and validate the optimal stimulation power and pulse length for each silicon nanowire size and cell type **[1]**.

4.3.1. SCREEN: screenshot1: 00:01-00:27 *Video Editor: please speed up*

4.4. Acquire a 2-10-second recording of the baseline intracellular calcium activity **[1]** before stimulating the nanowire with a single laser pulse of 1-10 milliwatts of power and a 1-10-millisecond duration, recording the resulting calcium wave for another 2-10 seconds **[1]**.

4.4.1. SCREEN: screenshot2: 00:22-00:56 *Video Editor: please speed up*

4.4.2. SCREEN: screenshot2: 01:21-01:43 *Video Editor: please speed up*

4.5. At the end of the experiment, transfer the recorded movies of the optical stimulation to the appropriate software program for additional analysis **[1]**.

4.5.1. Talent exporting data, with monitor visible in frame

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.4.-2.7., 4.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.4. Hitting the SiNW with the laser beam. The effect is very clear once it is done correctly.

Results

5. Results: Representative Intra- and Intercellular Electrical Calcium Coupling Analyses

5.1. Using standard phase contrast optics, confluent cells are easily viewed by light microscopy **[1]**. Silicon nanowires, however, are barely visible, making their locations impossible to define by this method **[2]**.

5.1.1. LAB MEDIA: Figure 1A Phase and Dark images *Video Editor: please emphasize cell(s) in Phase image*
5.1.2. LAB MEDIA: Figure 1A Phase and Dark images

5.2. Superimposition of light and dark field images **[1]**, however, allows visualization of the perinuclear arrangement of the light reflective silicon nanowires **[1]**.

5.2.1. LAB MEDIA: Figure 1A Phase and Dark images *Video Editor: please merge Phase and Dark images to obtain Figure 1A Merged image*
5.2.2. LAB MEDIA: Figure 1A Merged image *Video Editor: please emphasize SiNW(s)*

5.3. Confocal microscopy **[1]**, can be used to visualize fluorescent marker-stained cytosol **[2]** and plasma membranes **[3]**, making the intracellular location of the silicon nanowires more evident **[4]**.

5.3.1. LAB MEDIA: Figure 1B
5.3.2. LAB MEDIA: Figure 1B *Video Editor: please emphasize green signal in left image*
5.3.3. LAB MEDIA: Figure 1B *Video Editor: please emphasize red signal in left image*
5.3.4. LAB MEDIA: Figure 1B *Video Editor: please emphasize white signals in right images*

5.4. Using spinning disc confocal microscope, a brightfield image can be obtained to identify the location of the silicon nanowire to be stimulated **[1]**.

5.4.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize pink arrowhead/SiNW indicated by arrowhead in left image*

5.5. A short baseline video of the spontaneously beating cardiomyocytes and the resting myofibroblasts can then be acquired **[1]**.

5.5.1. LAB MEDIA: Supplementary Video 2: 00:00-00:03

5.6. After stimulation, the calcium propagation within the co-culture can recorded **[1]**.

5.6.1. LAB MEDIA: Supplementary Video 2: 00:04-00:19

5.7. The time at which the different cells become excited **[1]** can be determined by the point at which the change in average optical flow reaches its maximum **[2]**.

5.7.1. LAB MEDIA: Figure 3A top image
5.7.2. LAB MEDIA: Figure 3A bottom image

5.8. The optical flow can then be calculated to aid in the identification of the time of activation within each cell region **[1]**.

5.8.1. LAB MEDIA: Figure 3B *Video Editor: please emphasize t= equations and/or corresponding peaks in bottom graph*

5.9. The intra-cellular velocities can be determined for each cell using kymographs **[1]**, with the slope of the line representing the inverse of the intra-cellular propagation speed **[2]**.

5.9.1. LAB MEDIA: Figure 3C
5.9.2. LAB MEDIA: Figure 3C *Video Editor: please emphasize diagonal line in each graph*

5.10. For example, here a summary of the different inter- and intra-cellular velocities measured for the cell interactions within a single co-culture can be observed **[1]**.

5.10.1. LAB MEDIA: Figure 3D *Video Editor: please sequentially add/emphasize data bars from black to grey*

Conclusion

6. Conclusion Interview Statements

6.1. **Aleksander Prominski**: To achieve a focused spot on the SiNW, it is important to always make sure that the laser is properly collimated **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.3.)

6.2. **Menahem Y. Rotenberg**: This method can be utilized for in vivo studies by injecting the cell-silicon hybrids directly into the tissue and using ex vivo methods to study the electrical coupling in 3D **[1]**.

6.2.1. LAB MEDIA: To be provided by Authors by 19 Sep: Named talent says the statement above in an interview-style shot, looking slightly off-camera