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Title: Temperature-Controlled Assembly and Characterization of a Droplet Interface Bilayer

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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 something similar? **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. **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 videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

Current Protocol Length

Number of Steps: 34
Number of Shots: 47

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Andy Sarles:** This protocol enables researchers to precisely heat a droplet interface bilayer or DIB model membrane, which allows for studying a variety of temperature-related effects that occur in cellular environments. NOTE: 1.1 and 1.3 shot together

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.2*

1.2. **Jessie Ringley:** The core advantage stems from the ability to locally measure and control bath temperature in a low-volume reservoir without obstructing access for electrical or optical characterization of the DIB. NOTE: 1.2 and 1.4 shot together

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

OPTIONAL:

1.3. **Andy Sarles:** The ability to precisely heat the fluid bath unlocks the possibility to study the transport and signaling properties in DIBs formed from a much wider variety of membrane constituents, including natural cellular extracts.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.6.1*

1.4. **Jessie Ringley:** This is a multi-step protocol leveraging multiple equipment that must be followed closely to achieve sufficient experimental accuracy. Thus, any ambiguity in the written process can be clarified via visual demonstration.

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

Protocol

2. Heated Fixture Preparation

2.1. To begin, gather 2 pieces of 1-millimeter thick insulative rubber trimmed to 25 by 40 millimeters in length, 2 pieces of a 6-millimeter-thick rubber that are also 25 by 40 millimeters **[1]**, a prepared aluminum base fixture assembly **[2]**, and an acrylic oil reservoir that fits in the viewing window of the aluminum base fixture **[3]**.

2.1.1. WIDE: Talent gathering insulative rubber and two pieces of 6-millimtere thick rubber NOTE: 2.1.1 – 2.1.3 shot together

2.1.2. Aluminum base fixature assembly

2.1.3. Acrylic oil reservoir

2.2. Prepare the aluminum fixture by attaching to the bottom of the fixture to a glass coverslip viewing window with ultraviolet curable adhesive **[1]** and adhering 1 resistive heating element to the top of each 25-millimeter square side flange of the fixture **[2]**.

2.2.1. Talent attaching coverslip to the bottom of aluminum fixture NOTE: If shots 2.2 not recorded, delete the entire step

2.2.2. Talent attaching resistive heating element to the top of fixture

2.3. Place the thinner rubber pieces onto the stage of the microscope such that the long edge of each piece is tangential to the stage opening **[1]**. Position the aluminum-base fixture on top of the insulative pads with the viewing window of the fixture centered above the objective lens **[2]**.

2.3.1. Talent placing thinner rubber pieces onto the stage of microscope NOTE: 2.3.1 – 2.3.2 shot together

2.3.2. Talent placing fixture on top of insulative pads

2.4. Place a thicker piece of rubber on top of each resistive heating element **[1]**, then use a microscope stage clip to hold it in place to protect the heating elements from damage caused by the stage clips and to insulate against accidental electrical shorting **[2]**.

2.4.1. Talent placing a thicker piece of paper on top of resistive heating element NOTE: 2.4.1 – 2.4.2 shot together

2.4.2. Talent using microscope stage clip to hold it in place

2.5. Carefully bend the measurement-end of a thermocouple to achieve a 90-degree angle at about 4 millimeters from the end **[1]**. Insert the bent tip of the thermocouple into the lower left corner of the aluminum fixture and gently secure it with the locking screw **[2]**.

2.5.1. Talent bending the thermocouple NOTE: 2.5.1 – 2.5.2 shot together

2.5.2. Talent bending the thermocouple tip and locking it using screw

2.6. Place the acrylic reservoir into the well of the aluminum fixture prior to adding hexadecane oil to the well of the aluminum fixture to minimize the risk of trapping air bubbles between the viewing window and the bottom of the acrylic reservoir **[1]**.

2.6.1. Talent placing acrylic reservoir into the well of aluminum fixture

2.7. Dispense about 1,000 microliters of hexadecane oil into the well of aluminum fixture to provide a maximal surface area for heat transfer to occur, while not allowing oil to spill over the edges of the fixture onto the microscope stage or objective lens **[1]**.

2.7.1. Talent dispensing oil into the well of fixture *Videographer: This step is important!*
NOTE: 2.7.1 – 2.8.2 shot together

2.8. Dispense about 1,000 microliters of hexadecane oil into the acrylic reservoir without overfilling it **[1]**.

2.8.1. Talent dispensing oil into acrylic reservoir

3. Characterization of Temperature-Dependent Behaviors in DIBs

3.1. Measure the nominal capacitance of the membrane while lowering the temperature of the oil bath from a set point that permits bilayer formation to identify thermotropic phase transitions of the lipids in the membrane **[1]**.

~~3.1.1. Talent measuring the capacitance while lowering the temperature of oil bath~~
NOTE: Combine this VO with the next shot

3.2. Right click the **temperature graph** on the GUI and clear the displayed data to ensure that sufficient space in the buffer is available for subsequent recordings **[1]**.

3.2.1. SCREEN: clear chart step 3.2.mp4.0:00-0:07

3.3. Using the waveform generator connected to the patch clamp amplifier, apply a triangular voltage waveform across the Droplet Interface Bilayer, or DIB, electrodes **[1-TXT]** and record the induced current response through the bilayer **[2]**.

3.3.1. Talent applying triangular voltage waveform across the electrodes **TEXT: e.g., 10 mV, 10 Hz.** *Videographer: This step is important!*

3.3.2. SCREEN: starting current recording step 3.3.2.mp4. 0:00-0:18

3.4. Cool the bilayer by reducing the set point temperature in 5-degree increments, waiting a minimum of 5 minutes at the new steady state temperature between temperature changes until the desired temperature is achieved **[1]**.

3.4.1. Talent reducing the temperature

3.5. After the oil bath and cooling bilayer to the desired minimum temperature, right click the temperature graph in the GUI again and export the temperature data versus time to a spreadsheet software **[1]**. Stop the current recording **[2]**.

3.5.1. SCREEN: export to excel step 3.5.1.mp4. 0:00-0:22

3.5.2. SCREEN: stopping the current recording step 3.5.2.mp4.0:00-0:06

3.6. From the measured current, calculate the nominal capacitance of the square wave-current response versus time during the cooling period **[1]**.

3.6.1. SCREEN: calc nominal capacitance step 3.6.1.mp4.0:00-0:06 *Video editor: Speed up the alignment running*

3.7. Plot nominal capacitance versus temperature to observe how membrane capacitance changed, then locate nonmonotonic changes in capacitance versus temperature to identify melting temperature **[1]**.

3.7.1. SCREEN: plot nominal capacitance vs temp and identify melting temp step 3.7 and 3.8.mp4.0:00-0:15

3.8. Similarly, assess the quasi-static specific capacitance of the bilayer at fixed temperatures by successively incrementing the temperature of the oil bath and the bilayer area **[2]**.

~~3.8.1. Talent assessing quasi-static specific capacitance of bilayer~~

3.8.2. Talent incrementing the temperature

3.9. Change the set point temperature in 10-degree Celsius increments using the GUI and allow the system to equilibrate to the new temperature **[1]**.

3.9.1. SCREEN: changing set point temp step 3.9.1.mp4.0:00-0:06

3.10. Perform previously described steps to initiate the measurement of capacitive current and recording. Change the bilayer area by carefully adjusting the positions of the electrodes using the micro-manipulators **[1].**

3.10.1. Talent adjusting the bilayer area using micromanipulator

3.11. Allow for the square-wave current to reach a steady state amplitude and collect images of the DIB to enable calculation of membrane area versus time. Use a camera mounted to the microscope to image the bilayer as seen from the aperture **[1]**.

3.11.1. Talent collecting images of DIB

3.12. Simultaneously, add a digital tag in the current recording software to mark the corresponding timepoint for image collection **[1]**.

3.12.1. SCREEN: adding digital tag step 3.12.1.mp4.0:00-0:13

3.13. Obtain a total of 5 DIB images and steady-state regions of bilayer current, then reset the temperature and repeat the imaging **[1]**.

3.13.1. ~~Talent collecting the images of DIB~~ NOTE: Demonstrated in 3.11.1.

3.14. Analyze the current recordings and DIB images at the tagged timepoints corresponding to steady-state bilayer areas. Extract bilayer capacitance and area for each temperature **[1]**.

3.14.1. SCREEN: Extracting area step 3.14.1, capacitance same collection same as 3.6.1.mp4.0:00-0:11

3.15. Plot capacitance versus area for each temperature and compute the slope of a first-order regression, which represents the specific capacitance of the bilayer at each temperature **[1]**.

3.15.1. SCREEN: plotting specific capacitances vs temps and identifying melting temp steps 3.15.1, 3.16.1, 3.17.1.mp4.0:00-0:03

3.16. Then, plot values of specific capacitance versus their respective temperatures **[1]**.

3.16.1. SCREEN: plotting specific capacitances vs temps and identifying melting temp steps 3.15.1, 3.16.1, 3.17.1.mp4.0:05-0:10

3.17. Examine the specific capacitance versus temperature data for non-monotonic variations to identify melting temperatures **[1]**.

3.17.1. SCREEN: plotting specific capacitances vs temps and identifying melting temp steps 3.15.1, 3.16.1, 3.17.1.mp4.0:10-0:17

3.18. Assess the dynamics of voltage-dependent ion channel formation by generating a DC voltage step input across the bilayer **[1]**.

3.18.1. Talent generating DC voltage step input across the bilayer

3.19. Set **Initial Voltage** to the desired step value in millivolts **[1]** and the **Final Voltage** and **Step Size** to a value higher than the desired step **[2]**.

3.19.1. SCREEN: setting initial voltage, final voltage, and step size steps 3.19.1 and 3.19.2.mp4.0:00-0:03

3.19.2. SCREEN: setting initial voltage, final voltage, and step size steps 3.19.1 and 3.19.2.mp4.0:04-0:11

3.20. Set a desired duration time for the step input in seconds, then choose the desired polarity for the step input **[1]**.

3.20.1. SCREEN: setting step time and polarity steps 3.20.1 and 3.20.2.mp4.0:00-0:08

3.21. Switch the patch clamp amplifier to send the command voltage originating from LabView or voltage output module to the headstage **[1]**.

3.21.1. SCREEN: sending command voltage from labview step 3.21.1.mp4.0:00-0:05

3.22. Initiate current recordings. Turn on the voltage and record the induced current response, which should exhibit an S -shaped response to a critical voltage **[1-TXT]**.

3.22.1. SCREEN: recording induced current steps 3.23.1 and 3.24.1.mp4.0:00-0:05 **TEXT: e.g., ~70 mV for 1 µg/mL Mz in 2 mg/mL BTLE**

3.23. Separately, obtain dynamic current-voltage relationships for a membrane at desired temperatures to reveal voltage-dependent relationships, such as ion channel behaviors **[1]**.

3.23.1. SCREEN: recording induced current steps 3.23.1 and 3.24.1.mp4.0:06-0:11

3.24. Switch the patch clamp amplifier to send the command voltage originating from the waveform generator to the headstage and initiate current recordings **[1]**.

3.24.1. Talent sending the command voltage from waveform generator and initiating the current recordings NOTE: If there is no separate shot for this, combine 3.24 VO with the shot 3.25.1

3.25. On the waveform generator, output a continuous sinusoidal waveform with a desired amplitude, offset, and frequency. Record the induced current response across one ore multiple cycles and repeat as desired for different sine wave amplitudes, frequencies, and temperatures **[1]**.

3.25.1. SCREEN: Hysteresis screen cap for step 3.25.1.mkv. *Video Editor: Either just show the first 40 – 60s and speed it up or do a time lapse.*

Results

4. Results: Characterization of a Droplet Interface Bilayer Formed by Temperature Controlled Assembly

4.1. The temperature control system was used to showcase the temperature-dependence of a DIB formed from brain total lipid extract, or BTLE, lipids. Measurements of capacitive current and temperature versus time are shown during a heating cycle from room temperature to approximately 60 degrees Celsius **[1]**.

4.1.1. LAB MEDIA: Figure 5a

4.2. Changes in nominal capacitance versus temperature across one complete cooling-heating cycle after initial bilayer formation at 60 degrees Celsius were documented **[1]**.

4.2.1. LAB MEDIA: Figure 5b

4.3. Quasi-static measurements of specific capacitance at different temperatures can be used to identify lipid melting temperature. Plotting capacitance of the bilayer versus bilayer area allows for a linear regression, where the slope represents the value of specific capacitance **[1].**

4.3.1. LAB MEDIA: Figure 6a

4.4. The DIB image shows that when temperature is below the melting temperature, the membrane adopts a highly adhesive state, even under tension from stretched droplets caused by well-separated electrodes **[1]**.

4.4.1. LAB MEDIA: Figure 6c

4.5. The current versus voltage traces shown were obtained by applying sinusoidal membrane voltages and measuring the induced current at two different temperatures. The arrows and subsequent numbers aid in visualizing the successive quarters of the sinusoidal voltage with respect to time **[1]**.

4.5.1. LAB MEDIA: Figure 7a

4.6. The measured current density for a Monazomycin-doped BTLE membrane at the same voltage level and two different temperatures is shown here **[1].**

4.6.1. LAB MEDIA: Figure 7b

Conclusion

5. Conclusion Interview Statements

5.1. **Jessie Ringley:** It is crucial to dispense the hexadecane oil into the well of the aluminum fixture correctly. If this is done out of sequence or not carefully, air bubbles will form under the acrylic well, which will obstruct the bottom-up view of the DIB.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.7.1*

5.2. **Jessie Ringley:** The user must also remember to clear the data buffer in the temperature control software prior to each measurement to ensure full recording.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

5.3. **Jessie Ringley:** This procedure allows for characterizing biomimetic membranes across a range of temperatures, which is needed to study temperature-dependence of membrane structure and transport. In addition, this capability could be used to reveal nanoscale effects of other membrane-active species, such as biological ion channels and engineered nanomaterials.

5.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.22.1 and 3.23.1*