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Cover Letter

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We are submitting to Journal of Visualized Experiments a manuscript by J. S. Najem, G. J. Taylor, N. Armendarez, R. J. Weiss, M. S. Hasan G. S. Rose, C. D. Schuman, A. Belianinov, S. A. Sarles, and C. P. Collier, entitled, "*Assembly and characterization of biomolecular memristors consisting of ion channel-doped lipid membranes*." Here, we describe a protocol to assemble and characterize a biomolecular memristor (memory-resistor) obtained from an insulating lipid bilayer formed between two water droplets in oil. Brain-like neuromorphic computing networks have the potential to perform complex calculations in less time, with less energy, and in a smaller footprint than conventional computing technologies. Early neuromorphic circuits utilized analogue or software-controlled very-large-scale integration models, or hardware based on CMOS technology to perform brain-inspired operations. However, the brain is not a solid-state electronic device but, rather, a highly-interconnected and reconfigurable network of cells that communicate across nm-thick lipid membranes via chemically-selective proteins. As a result, the behaviors of state-of-the-art solid-state memristors are largely phenomenological and are thus intrinsically limited in their ability to emulate biological synaptic functions.

Here, we describe the process of assembling and characterizing biomolecular memristors consisting of a 5nm-thick lipid bilayer formed between lipid-functionalized water droplets in oil and doped with voltage-activated alamethicin peptides. This article focuses on key modifications of the droplet interface bilayer method essential for achieving consistent and optimal memristor performance. Specifically, we describe how to incorporate alamethicin peptides in lipid bilayer membranes, and the impact of each constituent on the overall response of the memristor. We also present protocols for characterizing memristors, including measurement and analysis of memristive current-voltage relationships obtained via cyclic voltammetry, and short-term plasticity and learning in response to step-wise voltage pulse trains.

Yours Sincerely,

C. Patrick Collier, Ph.D.

TITLE:

Assembly and Characterization of Biomolecular Memristors Consisting of Ion Channel-doped Lipid Membranes

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SUMMARY:

Soft, low-power, biomolecular memristors leverage similar composition, structure, and switching mechanisms of bio-synapses. Presented here is a protocol to assemble and characterize biomolecular memristors obtained from insulating lipid bilayers formed between water droplets

in oil. The incorporation of voltage-activated alamethicin peptides results in memristive ionic conductance across the membrane.

ABSTRACT:

The ability to recreate synaptic functionalities in synthetic circuit elements is essential for neuromorphic computing systems that seek to emulate the cognitive powers of the brain with comparable efficiency and density. To date, silicon-based three-terminal transistors and two-terminal memristors have been widely used in neuromorphic circuits, in large part due to their ability to co-locate information processing and memory. Yet these devices cannot achieve the interconnectivity and complexity of the brain because they are power-hungry, fail to mimic key synaptic functionalities, and suffer from high noise and high switching voltages. To overcome these limitations, we have developed and characterized a biomolecular memristor that mimics the composition, structure, and switching characteristics of biological synapses. Here, we describe the process of assembling and characterizing biomolecular memristors consisting of a 5 nm-thick lipid bilayer formed between lipid-functionalized water droplets in oil and doped with voltage-activated alamethicin peptides. While similar assembly protocols have been used to investigate biophysical properties of droplet-supported lipid membranes and membrane-bound ion channels, this article focuses on key modifications of the droplet interface bilayer method essential for achieving consistent memristor performance. Specifically, we describe the liposome preparation process and the incorporation of alamethicin peptides in lipid bilayer membranes, and the appropriate concentrations of each constituent as well as their impact on the overall response of the memristors. We also detail the characterization process of biomolecular memristors, including measurement and analysis of memristive current-voltage relationships obtained via cyclic voltammetry, as well as short-term plasticity and learning in response to step-wise voltage pulse trains.

INTRODUCTION:

It is widely recognized that biological synapses are responsible for the high efficiency and enormous parallelism of the brain due to their ability to learn and process information in highly adaptive ways. This coordinated functionality emerges from multiple, highly complex molecular mechanisms that drive both short-term and long-term synaptic plasticity[1-5]. Neuromorphic computing systems aim to emulate synaptic functionalities at levels approaching the density, complexity, and energy efficiency of the brain, which are needed for the next generation of brain-like computers[6-8]. However, reproducing synaptic features using traditional electronic circuit elements is virtually impossible[9], instead requiring the design and fabrication of new hardware elements that can adapt to incoming signals and remember information histories[9]. These types of synapse-inspired hardware are known as mem-elements[9-11] (short for memory elements), which, according to Di Ventra et al.[9,11], are passive, two-terminal devices whose resistance, capacitance, or inductance can be reconfigured in response to external stimuli, and which can remember prior states[11]. To achieve energy consumption levels approaching those in the brain, these elements should employ similar materials and mechanisms for synaptic plasticity[12].

To date, two-terminal memristors[13-15] have predominantly been built using complementary metal-oxide-semiconductor (CMOS) technology, characterized by high-switching voltages and

high noise. This technology does not scale well due to high power consumption and low density. To address these limitations, multiple organic and polymeric memristors have been recently built. However, these devices exhibit significantly slower switching dynamics due to time-consuming ion diffusion through a conductive polymer matrix[16,17]. As a result, the mechanisms by which both CMOS-based and organic memristive devices emulate synapse-inspired functionalities are highly phenomenological, encompassing only a few synaptic functionalities such as Spike Timing Dependent Plasticity (STDP)[18], while overlooking other key features that also play essential roles in making the brain a powerful and efficient computer, such as pre-synaptic, short-term plasticity[19].

Recently, we introduced a new class of memristive devices[12] featuring voltage-activated peptides incorporated in biomimetic lipid membranes that mimics the biomolecular composition, membrane structure, and ion channel triggered switching mechanisms of biological synapses[20]. Here, we describe how to assemble and electrically interrogate these two-terminal devices, with specific focus on how to evaluate short-term plasticity for implementation in online learning applications[12]. Device assembly is based on the droplet interface bilayer (DIB)[21] method, which has been used extensively in recent years to study the biophysics of model membranes[21] and membrane-bound ion channels[22-24], and as building blocks for the development of stimuli-responsive materials[25,26]. We describe the membrane assembly and interrogation process in detail for those interested in neuromorphic applications but have limited experience in biomaterials or membrane biology. The protocol also includes a full description of the characterization procedure, which is as important as the assembly process, given the dynamic and reconfigurable electrical properties of the device[27]. The procedure and representative results described here are foundations for a new class of low-cost, low-power, soft mem-elements based on lipid interfaces and other biomolecules for applications in neuromorphic computing, autonomous structures and systems, and even adaptive brain-computer interfaces.

PROTOCOL:

1. General Instructions and Precautions

1.1. Select suitable, undamaged measuring/mixing glassware (flasks, beakers, etc.) and other labware (spatulas, scoops, etc.) for use.

1.2. Handle glassware carefully to avoid damaging, and wear latex or nitrile gloves to avoid contaminating the glassware/labware with residues from fingertips and to protect your skin.

1.3. Clean chosen glassware/labware thoroughly using detergent solution and water by scrubbing with a soft bottle brush until clean and all residues are removed.

1.4. Rinse thoroughly with tap water and then with deionized (DI) water. Place on drying rack to air dry.

1.5. **Optional:** Rinse the cleaned glassware/labware with isopropyl (IPA, 99.5%) and place

under vacuum to evaporate all residual IPA to ensure they are free of any contaminants (~2 h). Remove from vacuum chamber and place in clean environment.

NOTE: Use lint-free wipes for wiping glassware and labware. Purchase and use sterile small glass vials and safe-lock tubes for materials preparation and sample storage. For further details on glassware cleaning and other laboratory standard operating procedures, refer to the JoVE Science Education Database[28].

2. Preparation of Aqueous Buffer Solution

2.1. Wearing latex or nitrile gloves, select an appropriate and clean glass container to prepare 50 mL of aqueous buffer (500 mM sodium chloride (KCl), 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0).

2.2. Using a digital, high precision mass balance and a clean spatula, dispense 1.86378 g of KCl onto clean weighing paper and then add to the glass container.

NOTE: The amounts of KCl and MOPS should vary depending on the desired volume and desired final concentrations.

2.3. Weigh 0.10463 g of MOPS and add to the glass container. Then, add 50 mL of DI water to the glass container and vortex thoroughly until KCl and MOPS are completely dissolved.

2.4. Store the buffer solution at room temperature and use when needed.

NOTE: While buffer solutions can be stored for relatively long periods of time, it is recommended to use freshly prepared buffer solutions for better and more consistent results.

3. Preparation of Liposomes

NOTE: Step 3.1 only applies if phospholipids are acquired as lyophilized powders, and therefore, may be skipped if the phospholipids are purchased in chloroform.

3.1. Dissolve 5 mg of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) or Brain Total Lipid Extracts (BTLE) lipids in 1 mL of chloroform in a 5 mL sterile glass vial.

3.2. While gently swirling, evaporate the chloroform under a gentle stream of dry nitrogen until a lipid film remains at the bottom of the vial.

3.3. Place the vial containing the lipid film under vacuum for 10-12 h to allow for complete removal of residual chloroform.

3.4. Remove the vial from the vacuum chamber and rehydrate the lipid film by adding 10 mL of the aqueous buffer solution prepared in Step 2 to achieve a final lipid concentration of 2

mg/mL.

3.5. Freeze (-20 °C) and completely thaw the lipid suspension six times to facilitate multilamellar liposome assembly.

NOTE: Let the mixture thaw at room temperature, never in a heated environment.

3.6. Using a commercially available extruder, extrude the liposome solution by forcing the complete lipid suspension through a 0.1 μm pore diameter track-etched membrane. Extrude the suspension 11 times in immediate succession to obtain unilamellar liposomes with diameters of c.a. 100 nm needed for proper lipid monolayer formation. Store the liposome suspension at 4 °C and use within 1 week of preparation. For simplicity, refer to the resulting liposome solution as "A".

NOTE: For the extrusion of BTLE liposomes, the researcher is encouraged to warm up the extruder to 45-50 °C, higher than the phase transition temperature of BTLE lipids (~ 37 °C)[23,29], to enable easier extrusion. Hydrated BTLE liposome suspensions can also be directly prepared (in place of freeze-thaw and extrusion) by placing the closed suspension vial into in a bath sonicator at 55 °C for ~15 min.

4. Reconstitution of Alamethicin Peptides

NOTE: This procedure describes the process of alamethicin reconstitution in liposomes to a final concentration of 1 μM. This concentration is sufficient to induce nA-level currents similar to those previously published[12]. Increasing the peptide concentration will reduce the switching threshold and increase the amplitudes of currents induced by applied voltage[29].

4.1. Dissolve alamethicin peptides in ethanol to a final concentration of 2.5 mg/mL, vortex briefly to mix well, and store the stock solution in freezer (-20 °C).

NOTE: Alamethicin peptides are usually purchased in powder form.

4.2. In a 1.5 mL safe-lock tube, mix 99 μL of solution "A" with 1 μL of alamethicin stock solution to achieve a final alamethicin concentration of 13 μM in the liposome suspension. Vortex to mix well. Refer to the resulting peptide-liposome solution as "B".

4.3. Mix 117 μL of solution "A" with 10 μL of solution B to achieve a final alamethicin concentration of 1 μM, and then vortex to mix well. Refer to the resulting solution as "C".

4.4. Store the solutions "B" and "C" at 4 °C and use as needed.

5. Preparation of Agarose gel

5.1. Using a digital, high precision mass balance and a clean spatula, add 0.5 g of agarose powder to a clean weighing paper.

5.2. Transfer weighed agarose to a 100-mL clean glass beaker and add 50 mL of DI water to the agarose.

NOTE: This will yield a 1% (wt/vol) agarose gel solution.

5.3. Place a clean stirring magnet inside the glass beaker and place the beaker on a stirring hot plate.

5.4. While stirring, bring the mixture to a boil until agarose is completely dissolved.

5.5. Remove the beaker from the hot plate to let the mixture cool to room temperature. Store at 4 °C and use when needed.

5.6. Before using again, re-melt the agarose by heating with a hot plate or microwave.

6. Fabrication of the Oil Reservoir

NOTE: The procedure described below is just one of many ways that an oil reservoir can be fabricated. The reader is encouraged to design and fabricate a reservoir based on available materials, machining capabilities, and specific needs.

6.1. Using a band saw, cut a 12 x 12 x 12 mm acrylic cube from a larger 12 mm thick acrylic sheet.

6.2. Mill a 12 mm diameter hole to a depth of 8-12 mm in the acrylic tube (**Figure 1a**).

7. Preparation of Electrodes

7.1. Using scissors, cut two pieces (75 mm) of silver wires (125 μm-diameter).

7.2. Using an open-flame lighter, melt one end of each silver wire to form small spherical balls (around 250 μm in diameter).

7.3. Immerse the ball ends in bleach for 1-2 h to create a silver silver-chloride (Ag/AgCl) coating. A dark gray color indicates that the Ag/AgCl coating has formed (**Figure 2a**).

7.4. Remove both wires from bleach, rinse thoroughly with DI water and place aside on a clean lint-free wipe.

7.5. Dip the ball ends into molten agarose gel to create a thin layer. This gel coating helps to anchor the aqueous droplets onto the wires under oil.

7.6. Using a glass cutter, split a 10-cm long, 1/0.58 OD/ID mm borosilicate glass capillary into two 5 cm capillaries.

7.7. Insert one of the glass capillaries into an electrode holder (**Figures 2b, c**), and then feed one of the Ag/AgCl wire into the glass capillary (**Figure 2d**). Feed the other Ag/AgCl wire into the second glass capillary.

7.8. Mount the second glass capillary to a glass micropipette holder (**Figures 2e, f**).

8. Setting Up the Experiment

8.1. Place a 1 mm thick, 25 x 75 mm glass slide on the stage of an inverted microscope (**Figure 1a**).

8.2. Dispense a few drops of hexadecane oil onto the center of the glass slide, and then place the oil reservoir directly onto the oil on the glass slide.

NOTE: Adding oil between the glass slide and oil reservoir is used to match the refractive index of the substrate to provide clearer and sharper images.

8.3. Completely fill the oil reservoir with hexadecane oil. Make sure the reservoir is positioned above the objective lens.

NOTE: Other hydrophobic oils may be used as well.

8.4. Connect the electrode holder to the headstage of a current amplifier. The headstage must be mounted on a micromanipulator (**Figure 1a**) to minimize electrode length and electrical noise.

8.5. Mount the glass micropipette holder with the second Ag/AgCl wire onto another micromanipulator (**Figure 1a**).

8.6. Using the manipulators, position the electrodes such that the agarose coated tips of the Ag/AgCl wires are fully submerged into the oil reservoir at a similar vertical plane.

8.7. Align the two electrodes and separate them by a few millimeters (**Figures 1a, b**).

NOTE: After adding the droplets (described in **Step 13**), the wires must be brought all the way down until the electrode tips are touching the bottom of the oil reservoir. This step will ensure that the wires do not oscillate, and thus, will minimize unnecessary fluctuations in the measured current.

9. Proper Grounding to Reduce Electrical Noise

9.1. Create a **Ground bus** by threading a screw into the anti-vibration table on which the microscope is placed (**Figure 3a**).

NOTE: Using an anti-vibration table is required to minimize vibrations from the surrounding, which might cause undesired fluctuations in measured current.

9.2. Using a conductive wire, connect the screw to an earth ground (**Figure 3a**), and then connect the microscope stage to the **Ground bus**.

9.3. Place a Faraday cage over the experimental setup to reduce noise and then electrically connect it to the **Ground bus** (**Figure 3b**).

NOTE: It is always recommended to avoid unnecessary ground loops, as they may lead to an increase in measurement noise level.

10. Feedback-Controlled Heating

10.1. Machine an aluminum heating shell in which the oil reservoir can snugly fit[29].

10.2. Make sure to leave an opening at the bottom of the shell to be able to view through the shell via the inverted microscope.

10.3. Place a 30 x 30 mm resistive polyimide flexible heating element underneath the aluminum shell.

10.4. Place an insulating polydimethylsiloxane (PDMS) wafer beneath the heater to reduce heat loss in the downward direction and protect the microscope stage.

10.5. Insert a thermocouple into the oil phase. After making sure the thermocouple does not touch either Ag/AgCl wire, connect the thermocouple wires to a thermocouple data acquisition board and record temperature using custom programming software.

NOTE: Write an On-Off, proportional integral (PI) feedback temperature control to enable heating and passive cooling of the oil temperature to a desired value. Codes can be provided to readers upon request.

11. Setting Up the Software and Equipment

11.1. Prepare the data acquisition software by powering on computer(s), microscope, function generator, current amplifier, and low-noise data acquisition systems.

NOTE: While any current sensing equipment may be used, the following instructions are specifically for the one listed in **Table of Materials**. Researchers who wish to build their own current amplifier can refer to Shlyonsky et al.[30].

11.2. On the front panel of the patch clamp current amplifier, set the front panel display and source-measurement **Mode** dials to VHOLD/IHOLD and V-CLAMP, respectively.

11.3. On the front panel, set the **Lowpass** *Bessel Filter* to 1 kHz and **Output Gain** to 0.5.

NOTE: Choosing a low output gain enables recording larger higher current amplitudes, whereas increasing the gain sacrifices measurement range for reduce measurement noise.

11.4. Set the **Configuration** to WHOLE CELL β = 1. This value may be later switched to 0.1 to allow recording of larger amplitude currents.

11.5. Set all other control dials to zero or in a neutral position.

11.6. Initialize the software by double-clicking on the icon of the desktop.

11.7. Click **Configuration | Digitizer** to open the **Digitizer** dialog, and then click the **Change** button.

11.8. In the **Change Digitizer** dialog, select the appropriate digitizer from the **Digitizer Type** list.

11.9. Click the **Scan** button to detect the digitizer.

11.10. Click **OK** to exit the **Change Digitizer** dialog, and then click ***OK*** to exit the ***Digitizer*** dialog.

11.11. Click **Configure | Lab Bench**.

11.12. In the **Input Signals** tab of the **Lab Bench**, set the scale factor to 0.0005 V/pA.

NOTE: This value must be updated if the gain or β values are changed.

12. Pipette Offset

NOTE: The procedure described below applies only to current amplifier mentioned in **Table of Materials**.

12.1. Using a micropipette, deposit 200 nL of the aqueous lipid solution “A” onto the ends of each Ag/AgCl wire under oil.

12.2. Bring the droplets into contact and press the **ZAP** button on the front panel of the amplifier to coalesce the droplets into one volume spanning both electrodes. This should induce a short-circuit response.

12.3. Set source-measurement mode dial to **TRACK**.

12.4. Change the front panel display dial to $\mathbf{V_{TRACK}}$.

12.5. Turn the **PIPETTER OFFSET** dial (clockwise or counterclockwise) until meter reads 0 mV and is stable.

12.6. Return the source-measurement mode dial to **V-CLAMP** and the front panel display dial to $\mathbf{V_{HOLD}/I_{HOLD}}$.

13. Formation of the Lipid Bilayer

13.1. Release the droplets that were previously deposited by moving the electrodes vertically out of the oil phase. This causes the droplets to fall from the electrodes into the oil. Re-submerge and position the electrodes in oil.

13.2. Use the micropipette to deposit 200 nL of lipid solution "A" on each of the wires.

13.3. Wait for 3-5 min to allow for spontaneous lipid monolayer assembly to occur at each water/oil interface.

NOTE: As the lipid monolayer forms, the water/lipid/oil interface surface tension decreases, which can cause the droplets to sag if the surrounding oil is sufficiently less dense[21].

13.4. Lower the electrodes (and droplets) until the ends of both electrodes barely touch the bottom of the oil reservoir (**Figure 1b**), and then move them horizontally to bring the droplets into contact.

NOTE: The lipid bilayer will spontaneously thin by excluding excess oil from between the contacting droplets. Typically, this process occurs within 1 min.

14. Electrical Characterization of the Biomolecular Memristor

14.1. Lipid Bilayer Formation

14.1.1. To record the lipid bilayer formation, which corresponds to an increase in electrical capacitance between droplets, apply a 10 Hz, 10 mV triangular waveform voltage using a function generator (**Figure 4**) connected to the external input of the patch clamp amplifier.

NOTE: Due to the capacitive nature of the lipid membrane, the resulting current response should be a square waveform (**Figure 4**). During the lipid bilayer formation, Step 11.6, the researcher should see a growth in the peak-to-peak current amplitude and also observe a visual change between connected droplets (**Figure 4**).

14.2. Current-voltage measurements

NOTE: The biomolecular memristor is modeled as a resistor and a capacitor in parallel[12,21]. Therefore, the current response of the device can contain both resistive and capacitive components depending on the frequency of the applied voltage. To study the memristive nature of the device, and to obtain the pinched hysteretic current-voltage relationship[12], it may be necessary to subtract capacitive current from the total current. The protocol below describes this procedure.

14.2.1. Using a function generator, apply a voltage waveform (triangular or sinusoidal) to an alamethicin-free lipid membrane assembled with droplets of solution "A".

14.2.2. Record the induced current response across multiple frequencies.

NOTE: Capacitive currents are minimized at frequencies below 10 mHz.

14.2.3. Record the size of the interfacial lipid bilayer by either measuring the diameter of the lipid membrane on computer, or by recording the peak-to-peak current amplitude resulting from the 10 Hz, 10 mV triangular wave. The current amplitude is proportional to the membrane capacitance, which in turn is proportional to the area of the membrane.

14.2.4. Remove the droplets that contain no alamethicin.

14.2.5. Add new aqueous droplets using solution "C" and form a lipid bilayer.

14.2.6. Use the micromanipulators to adjust contact between droplets such that the bilayer has a similar area (diameter or square-wave current amplitude) as the one formed earlier.

14.2.7. Repeat steps 12.1.1 and 12.1.2.

14.2.8. Subtract current recorded in step 12.1.2 from current recorded in step 12.1.6.

14.2.9. Plot the induced current versus applied voltage for each frequency and waveform to obtain the "pinched hysteresis" memristive response.

14.3. Pulse experiments

14.3.1. Using a custom programming software and analog voltage source, generate voltage pulses with specific high and low amplitudes, ON time, and OFF time.

NOTE: This is not needed if the voltage pulses could be generated using a commercial function generator.

14.3.2. Record the current in response to applied pulses.

14.3.3. Due to the capacitive nature of the memristor, capacitive spikes will be recorded. Remove spikes by applying a low-pass filter with appropriate passband.

REPRESENTATIVE RESULTS:

Figure 1 displays the experimental setup used to assemble and characterize the biomolecular memristor. Lowering the free ends of the electrodes to the bottom of the oil reservoir, as shown in **Figure 1b**, was found helpful to minimize vibrations of the electrodes and droplets that can result in variations in measured current and bilayer area, especially in cases where heating the oil can generate convective flow in the oil. **Figure 2** shows the procedure and result of assembling the Ag/AgCl wires, class capillaries, and electrode and micropipette holders. The setup is housed within a properly grounded Faraday cage (**Figure 3**) to minimize electromagnetic interference.

It is imperative to form a stable, insulating lipid bilayer for this study. In this protocol, a lipid monolayer assembles at the oil/water interfaces of the aqueous droplets immersed in oil. Upon contact between droplets, excess oil is excluded, and the opposing lipid monolayers thin to a 5-nm thick lipid bilayer. The most common technique used in bilayer electrophysiology is voltage-clamp, where the voltage across the bilayer is controlled and the induced current is measured. **Figure 4a** portrays the capacitive square-wave current induced by a 10 mV, 10Hz voltage during bilayer formation. While the amplitude increases upon the start bilayer thinning and subsequent radial expansion of the thinned membrane, the waveform remains square. Using the steady-state amplitude of square wave current, the nominal area of the lipid bilayer can be calculated using a predetermined value of specific membrane capacitance for a DPhPC bilayer[21]. Also, the bilayer area can be visually assessed by measurement of the bilayer diameter from an image taken with the microscope **Figure 4b**. For accurate lipid bilayer area calculations, the reader should refer to Taylor, et al.[21]. The area of the lipid bilayer can be adjusted by changing the relative positions of the droplets[21,31].

Upon application of a voltage bias to an alamethicin-free lipid bilayer, the current response will vary based on the frequency of the input voltage. At low frequencies (<10-50 mHz), where the resistance of the bilayer dominates the complex impedance, the ohmic current response is negligible because the nominal membrane resistance is typically greater than 10 GΩ. As the input frequency increases, membrane capacitance contributes more to the impedance of the system, resulting in the non-zero current response displayed in the plot of current versus voltage in **Figure 5a**. When the same input voltage waveform (150 mV) is applied to a biomolecular response consisting of an alamethicin-doped lipid membrane, and when the voltage amplitude surpasses a critical insertion threshold (~ 100 mV for a DPhPC membrane at room temperature), alamethicin peptides residing at the surface of the lipid bilayer insert into the membrane and aggregate to form conductive pores. The threshold-dependent formation of ion channels results in a nonlinear macroscopic current response, with exponentially increasing currents at voltages higher than the insertion threshold (**Figure 5b**). While alamethicin peptides are known to form rectifying ion channels only at sufficiently positive voltages, the symmetric nature of these current responses at both polarities is due to the insertion and aggregation of separate populations of peptides, each from opposite sides of the membrane. Depending on the frequency of the applied voltage, the induced current response may also contain contributions from the

capacitive current. Therefore, the capacitive current in **Figure 5a** must be subtracted from the total current displayed in **Figure 5b** to obtain only the memristive pinched hysteresis current-voltage response, displayed in **Figure 5c, d**.

Figure 6 displays the dynamic switching response of a biomolecular memristor induced by a voltage pulse train (130 mV (HIGH), 20mV (LOW), 100 ms (ON), 20 ms (OFF)). The OFF voltage is chosen to be 20 mV to differentiate the return of the device to an insulating state as alamethicin channels leave the bilayer rather from current simply vanishing at zero-voltage input. The cumulative increase in ON-state current during successive voltage pulses represents paired-pulsed facilitation, a plasticity that volatile biomolecular memristors are capable of exhibiting[12].

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental setup and main parts. (**a**) The standard workstation for assembling and characterizing the biomolecular memristor includes an inverted microscope, 3-axis micromanipulators, a digital camera, a vibration isolation table, an electrode holder, a glass micropipette holder, a current amplifier, a function generator, and an oil reservoir. The setup is assembled on the stage of the microscope as described in Steps 11-13. (**b**) A zoomed-in photograph of the setup showing the tips of the Ag/AgCl wires touching the bottom of the oil reservoir.

Figure 2: Electrode preparation procedure. Photographs showing: (**a**) the silver wires soaking in bleach; (**b**) an electrode holder; (**c**) a 5 cm long glass capillary connected to the electrode holder; (**d**) a Ag/AgCl electrode fed through the glass capillary; (**e**) a glass micropipette holder; and (**f**) the fully assembled electrodes and holders.

Figure 3: Grounding procedure. Photographs showing: (**a**) a screw threaded into the vibration isolation table surface to create a **Ground bus** when connected to earth ground; and (**b**) a lab-made Faraday cage covering the oil reservoir and electrode setup to shield the measurement from electromagnetic interference. Both the cage and microscope stage are tied to the **Ground bus** via cables I and II.

Figure 4: Real-time current measurements show initial bilayer thinning and areal growth. (**a**) The current measured (top) during spontaneous bilayer formation between lipid-coated droplets in response to a triangular waveform voltage. The magnitude of the measured current is directly proportional to the capacitance of the interface and hence, the area of the bilayer. The area of the interface can be varied by changing the distance between the two droplet-bearing electrodes. (**b**) An image acquired through the inverted microscope shows a bottom view and dimensions of a typical membrane-based biomolecular memristor.

Figure 5: Current-voltage relationship and pinched hysteresis. (**a**) The current-voltage responses of an alamethicin-free DPhPC lipid bilayer. A lipid-only membrane is highly insulating (~10 GΩ), which explains the low ohmic current response at 0.017 Hz, a frequency where the impedance is dominated by membrane resistance. At higher frequencies, membrane capacitance contributes more significantly to the total impedance of the interface, resulting in a non-zero induced

capacitive current. (**b**) The dynamic current-voltage relationships versus frequency of a lipid bilayer formed between two droplets containing alamethicin peptides (obtained with a triangular input wave). (**c**) The memristive, pinched hysteretic current response of the device is obtained by subtracting the capacitive current displayed in **a** from the total current displayed in **b**. (**d**) Zooming-in to highlight the differences between the total and the memristive currents.

Figure 6: Response of the biomolecular memristors to rectangular voltage pulses and plasticity. The device responds to subsequent voltage pulses with an increase in conductance during the ON time, despite intermittently restoring an insulating state during each OFF time. The increase in current from pulse to pulse shows that the instantaneous conductance of the device is a function of both the present stimulus and prior stimuli, analogous to short term plasticity in bio-synapses.

DISCUSSION:

This paper presents a protocol for assembling and characterizing biomolecular memristors based on ion channel-doped synthetic biomembranes formed between two droplets of water in oil. The soft-matter, two-terminal device is designed and studied to: 1) overcome constraints that are associated with solid-state technology, such as high noise, high energy consumption, and high switching voltages, 2) more closely mimic the composition, structure, switching mechanisms of biological synapses, and 3) explore the mechanisms and features of bio-synapse plasticity that are not exhibited by solid-state devices.

The droplet interface bilayer technique[21], which represents the building block of the present technology[12], is a simple, modular approach for membrane assembly that has been extensively used to study membrane biophysics[21], proteins[22], ion channels[29], and other biomolecules[32]. It offers specific advantages for precisely controlling and interrogating model membranes, and represents a building block for stimuli-responsive and autonomous materials[26]. Multiple methods have been developed to assemble droplet interface bilayers, including the hanging drop[21] method which was adapted as the main method to develop and characterize the biomolecular memristor. Even though this membrane assembly technique was used in previous studies, here we present a thorough protocol that allows researchers to recreate and study memristive droplet interface bilayers in their own laboratories. The protocol is specifically written in a way to allow researchers in non-membrane biology fields, such as the neuromorphic community, to understand and recreate these procedures.

In its simplest form, the protocol we have described herein for assessing memristive functionalities of a biomembrane can be replicated with basic laboratory equipment such as a function generator, a microscope, and a current measuring system. The assembled device is electrically equivalent to a resistor (~10 GΩ) and a capacitor wired in parallel. In the presence of peptides, such as alamethicin, that are capable of forming voltage-dependent pores in the membrane, the membrane resistance significantly drops, and resistive current can be detected in response to input voltage signals (DC or AC). However, the large membrane resistance and frequency-dependent electrical impedance of the device mean that: 1) induced currents are small (pA-nA), and subject to electromagnetic interference; and 2) care must be taken to

accurately induce and measure the desired memristive properties separate from capacitive membrane responses, respectively. In response to an AC voltage, and depending on the frequency of the signal, the recorded current will contain both capacitive and resistive components. To achieve the pinched hysteresis, which is a signature of memristive device, one must follow the protocol described in Step 14. The hanging wires are susceptible to vibrations, which can result in artefactual responses such as oscillations mistakenly attributed to the actual dynamics of the device. Positioning the wires at the bottom of the oil reservoir ameliorates this behavior.

The biomolecular memristor with its current structure and design emulates the short-term synaptic plasticity that occurs in the presynaptic terminal. It also mimics some of the mechanisms that cause presynaptic paired pulsed facilitation in the brain due to the accumulation and depletion of neurotransmitter vesicles in the presynaptic neuron. This methodology for assembling synaptic mimics enables the study and validation of biomimetic processes responsible for many types of short-term plasticity, and the optimization of modularity and scalability not possible with other technologies[33]. Unforeseen functionality may be discovered by either modifying the membrane composition, the types of ion channels that are incorporated into the membrane, and even the number of connected droplets and interfacial membranes constituting each two-terminal device. As an example, we have recently demonstrated the online learning capabilities of the biomolecular memristor by interfacing it with a solid-state neuron[34].

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DISCLOSURES:

REFERENCES:

1 Thompson, R. F. The neurobiology of learning and memory. *Science.* **233** (4767), 941-947, (1986).

2 Squire, L. R. Memory systems of the brain: a brief history and current perspective. *Neurobiology of learning and memory.* **82** (3), 171-177, (2004).

3 Benfenati, F. Synaptic plasticity and the neurobiology of learning and memory. *Acta Bio Medica Atenei Parmensis.* **78** (1Suppl), 58-66, (2007).

4 Marx, G. & Gilon, C. The molecular basis of memory. *ACS Chemical Neuroscience*. **9** (8), 633-642, (2012).

5 Izquierdo, I. & Medina, J. H. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiology of learning and memory.* **68** (3), 285-316, (1997).
6 Merolla, P. A. et al. A million spiking-neuron integrated circuit with a scalable communication network and interface. *Science.* **345** (6197), 668-673, (2014).
7 Benjamin, B. V. et al. Neurogrid: A mixed-analog-digital multichip system for large-scale neural simulations. *Proceedings of the IEEE.* **102** (5), 699-716, (2014).
8 Furber, S. Large-scale neuromorphic computing systems. *Journal of neural engineering.* **13** (5), 051001, (2016).
9 Di Ventra, M. & Pershin, Y. V. The parallel approach. *Nature Physics.* **9** (4), 200-202, (2013).
10 Chua, L. Memristor-the missing circuit element. *IEEE Transactions on circuit theory.* **18** (5), 507-519, (1971).
11 Di Ventra, M., Pershin, Y. V. & Chua, L. O. Circuit elements with memory: memristors, memcapacitors, and meminductors. *Proceedings of the IEEE.* **97** (10), 1717-1724, (2009).
12 Najem, J. S. et al. Memristive Ion Channel-Doped Biomembranes as Synaptic Mimics. *ACS Nano.* (2018).
13 Strukov, D. B., Snider, G. S., Stewart, D. R. & Williams, R. S. The missing memristor found. *Nature.* **453** (7191), 80-83, (2008).
14 Prezioso, M. et al. Training and operation of an integrated neuromorphic network based on metal-oxide memristors. *Nature.* **521** (7550), 61-64, (2015).
15 Prodromakis, T., Toumazou, C. & Chua, L. Two centuries of memristors. *Nature Materials.* **11** (6), 478, (2012).
16 Berzina, T. et al. Optimization of an organic memristor as an adaptive memory element. *Journal of Applied Physics.* **105** (12), 124515, (2009).
17 van de Burgt, Y., Melianas, A., Keene, S. T., Malliaras, G. & Salleo, A. Organic electronics for neuromorphic computing. *Nature Electronics.* 1, (2018).
18 Dan, Y. & Poo, M.-M. Spike timing-dependent plasticity: from synapse to perception. *Physiological reviews.* **86** (3), 1033-1048, (2006).
19 Zucker, R. S. & Regehr, W. G. Short-term synaptic plasticity. *Annual Reviews of Physiology.* **64** (1), 355-405, (2002).
20 Shepherd, J. D. & Huganir, R. L. The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annual Review of Cell Developmental Biology.* **23** 613-643, (2007).
21 Taylor, G. J., Venkatesan, G. A., Collier, C. P. & Sarles, S. A. Direct in situ measurement of specific capacitance, monolayer tension, and bilayer tension in a droplet interface bilayer. *Soft Matter.* **11** (38), 7592-7605, (2015).
22 Najem, J. S. et al. Activation of bacterial channel MscL in mechanically stimulated droplet interface bilayers. *Scientific Reports.* **5** 13726, (2015).
23 Taylor, G. J. et al. Capacitive Detection of Low-Enthalpy, Higher-Order Phase Transitions in Synthetic and Natural Composition Lipid Membranes. *Langmuir.* **33** (38), 10016-10026, (2017).
24 Taylor, G. et al. Electrophysiological interrogation of asymmetric droplet interface bilayers reveals surface-bound alamethicin induces lipid flip-flop. *Biochimica et biophysica acta (BBA)-Biomembranes.* (2018).
25 Sarles, S. A., Garrison, K. L., Young, T. T. & Leo, D. J. Formation and Encapsulation of Biomolecular Arrays for Developing Arrays of Membrane-Based Artificial Hair Cell Sensors.

Proceedings of the Asme Conference on Smart Materials, Adaptive Structures and Intelligent Systems (Smasis 2011), Vol 2. 663-671, (2012).
26 Sarles, S. A. & Leo, D. J. Membrane-based biomolecular smart materials. *Smart Materials & Structures.* **20** (9), (2011).
27 Sarles, S. A. *Physical encapsulation of interface bilayers*, Virginia Tech, (2010).
28 JoVE Science Education Datatbase. *Organic Chemistry II.* Cleaning Glassware. *Journal of Visualized Experiments*, Cambridge, MA, (2018).
29 Taylor, G. J. & Sarles, S. A. Heating-enabled formation of droplet interface bilayers using Escherichia coli total lipid extract. *Langmuir.* **31** (1), 325-337, (2014).
30 Shlyonsky, V., Dupuis, F. & Gall, D. The OpenPicoAmp: an open-source planar lipid bilayer amplifier for hands-on learning of neuroscience. *Plos One.* **9** (9), e108097, (2014).
31 Najem, J. S. et al. Multifunctional, Micropipette-based Method for Incorporation And Stimulation of Bacterial Mechanosensitive Ion Channels in Droplet Interface Bilayers. *Journal of Visualized Experiments.* 10.3791/53362 (105), (2015).
32 Bayley, H. et al. Droplet interface bilayers. *Molecular Biosystems.* **4** (12), 1191-1208, (2008).
33 Nguyen, M., Srijanto, B., Retterer, S., Collier, C. P. & Sarles, S. A. Hydrodynamic trapping for rapid assembly and in situ electrical characterization of droplet interface bilayer arrays. *Lab on a Chip.* **16**, 3576-3588 (2016).
34 Weiss, R. et al. in *Biomedical Circuits and Systems Conference.* Accepted.

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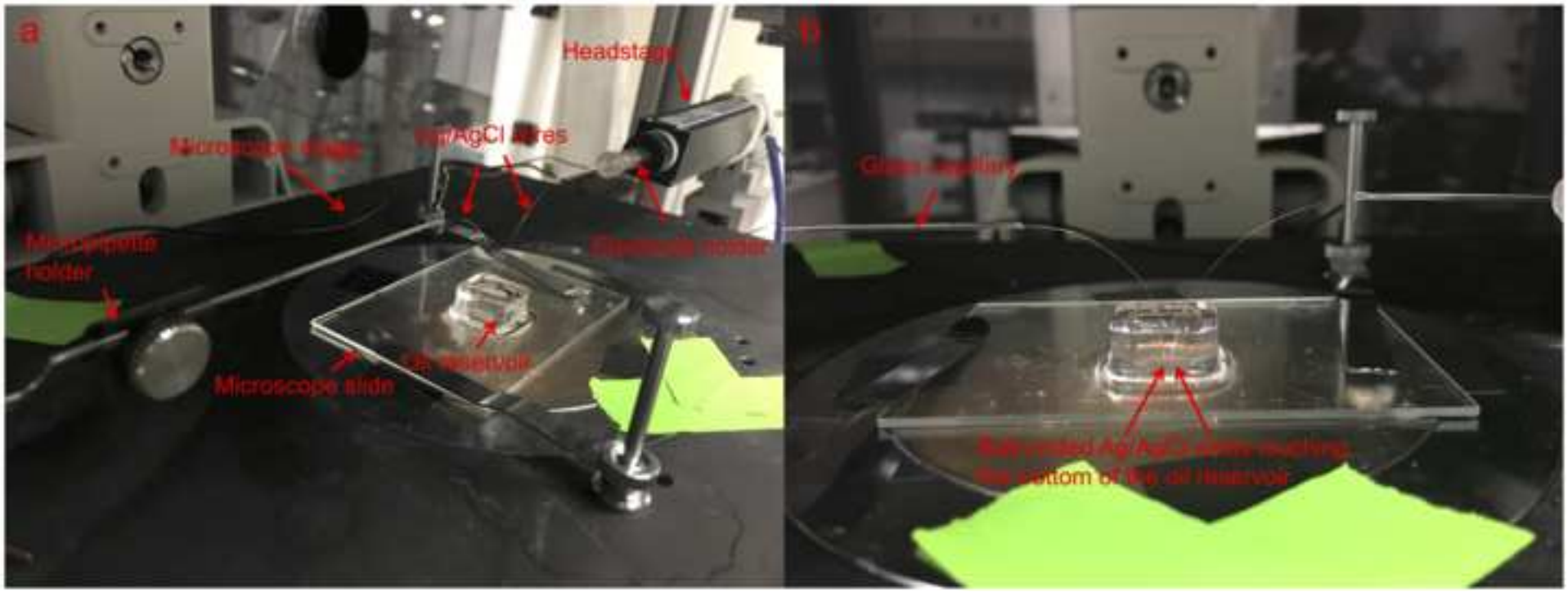

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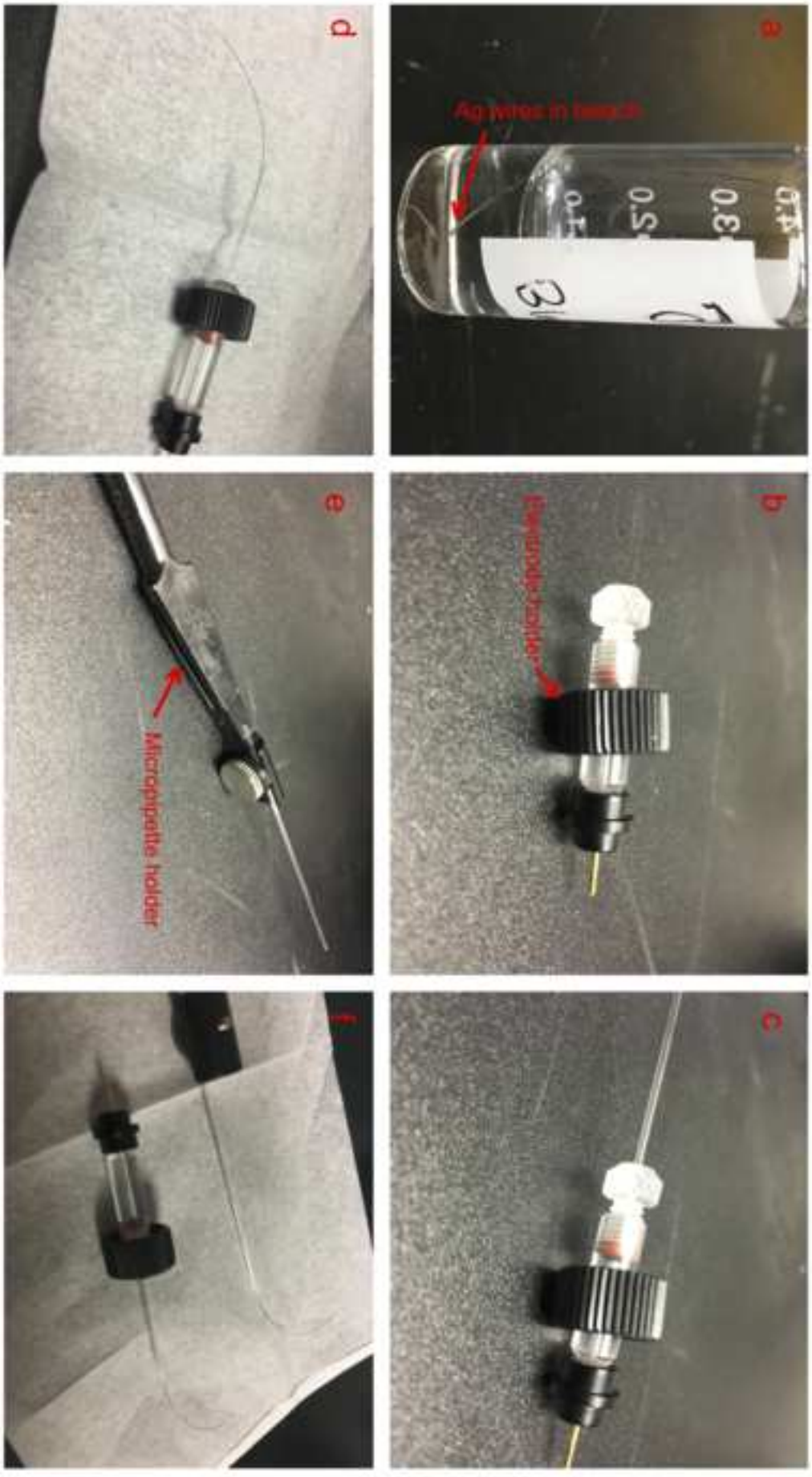

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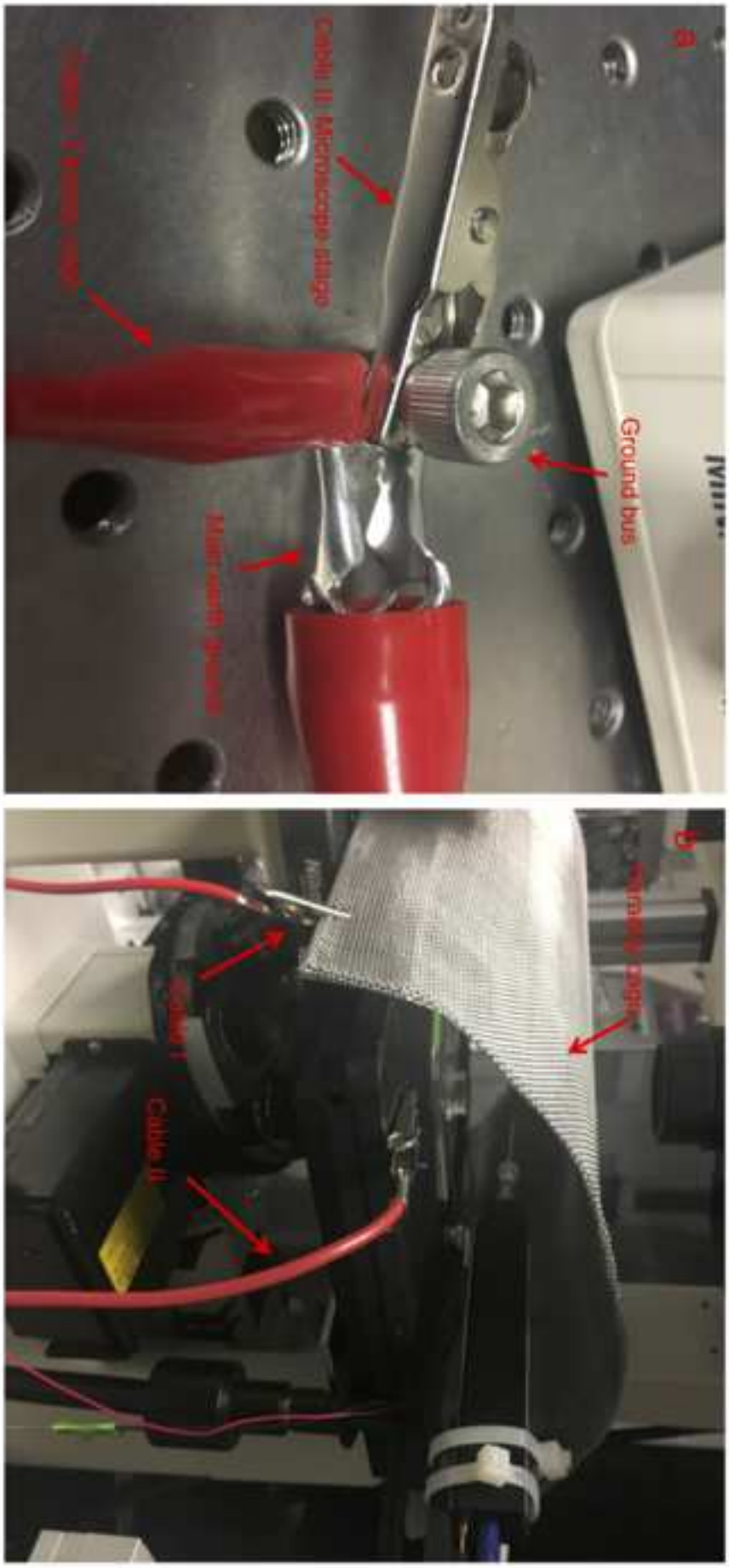

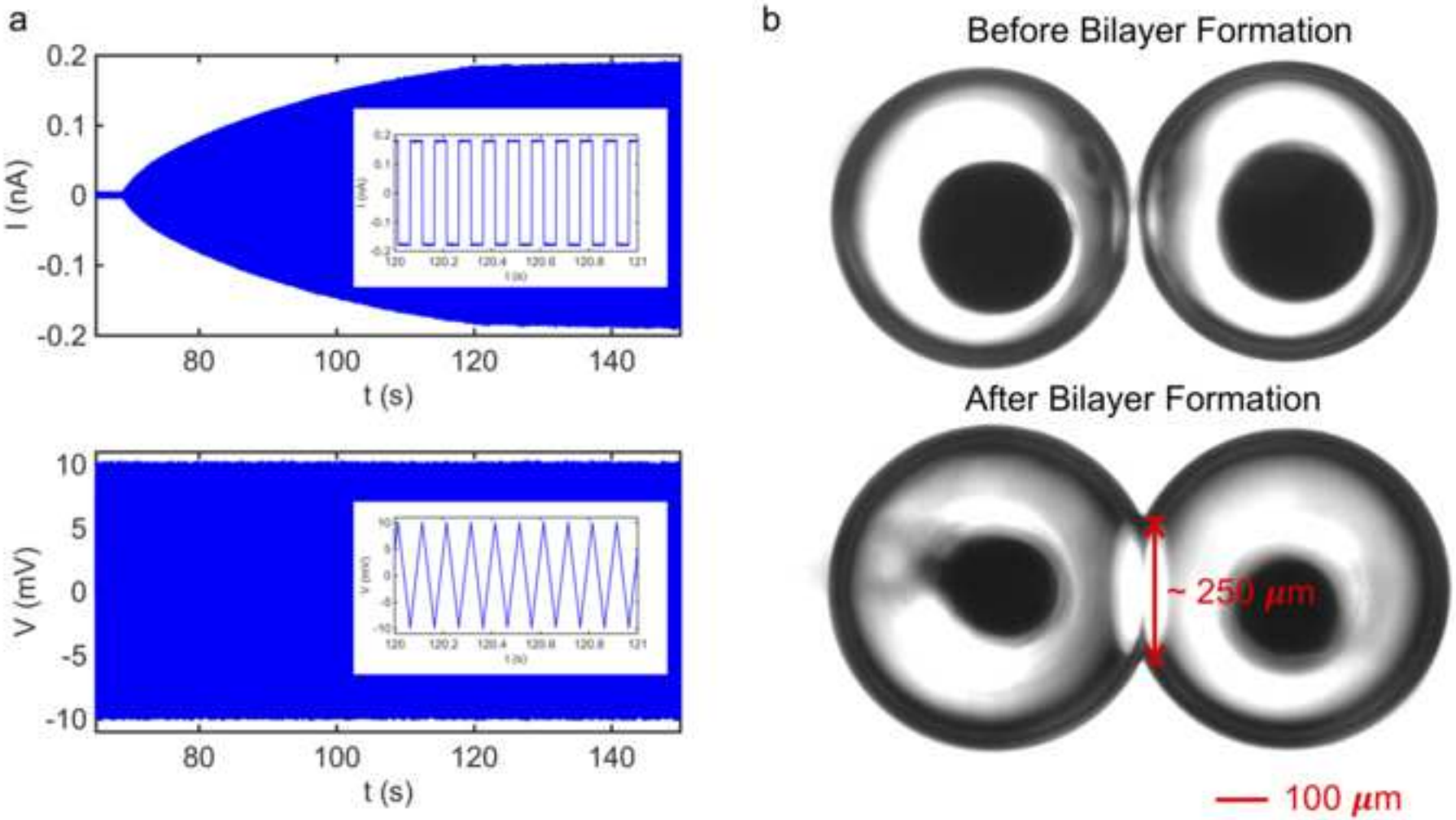

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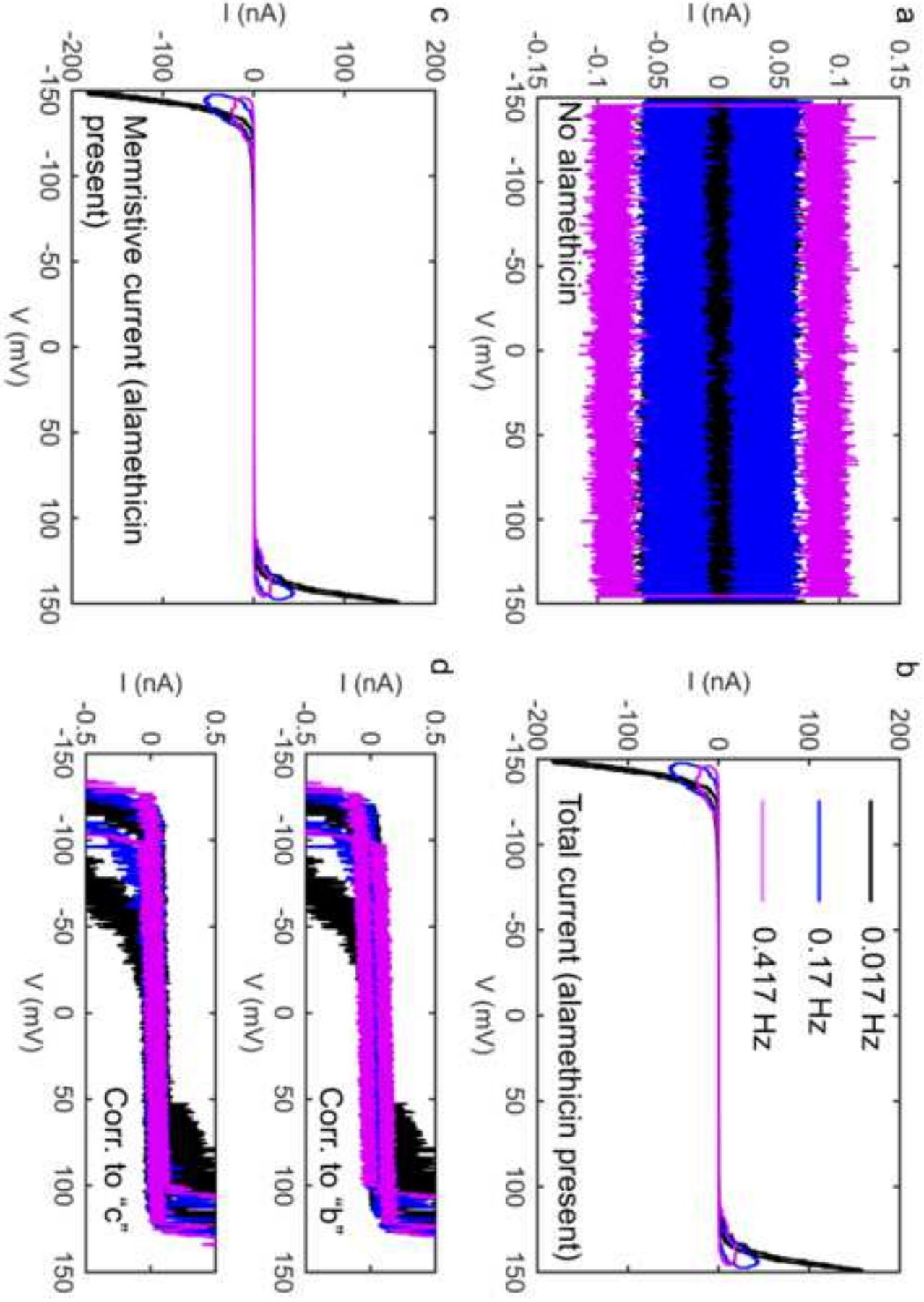

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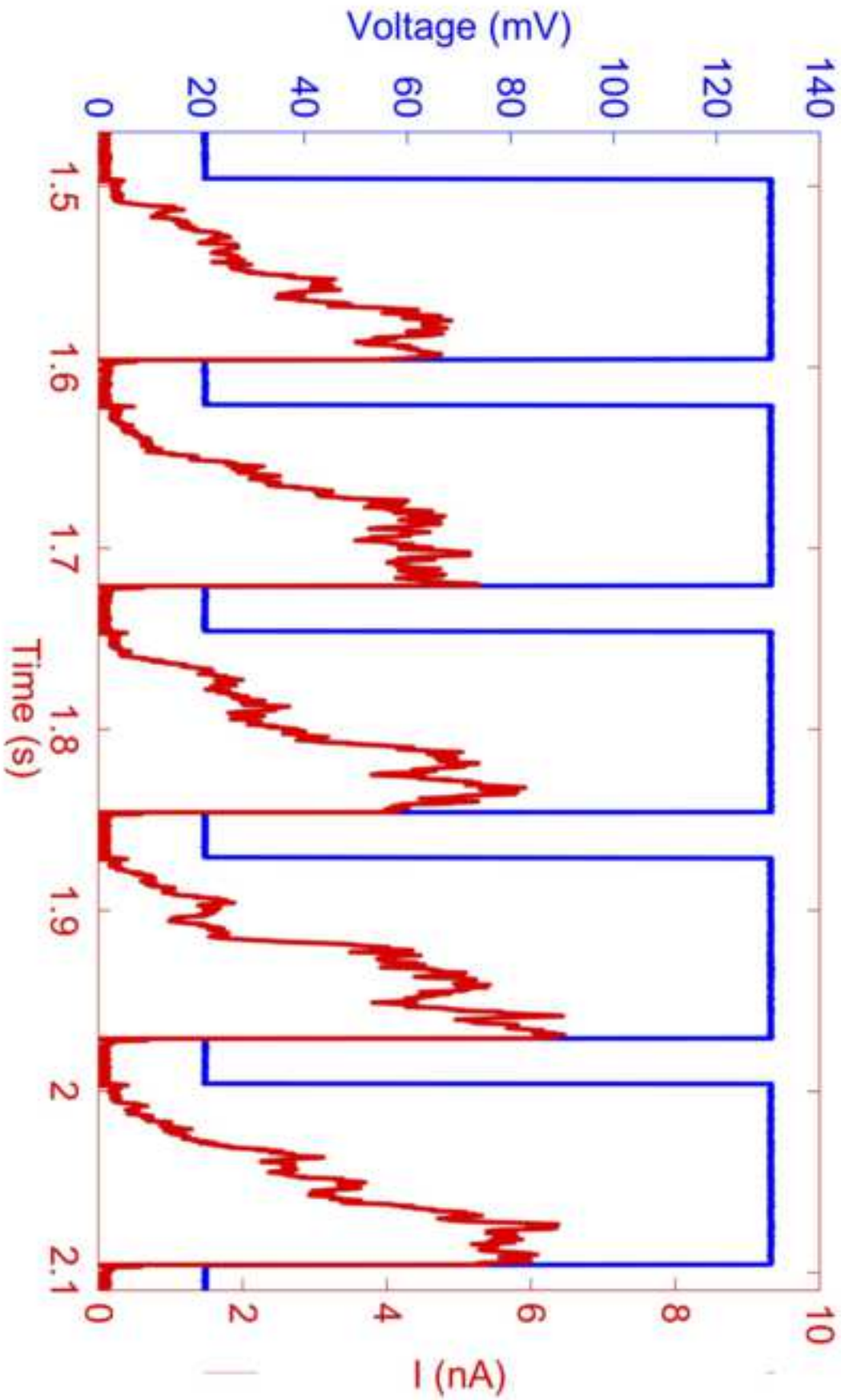

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1,2-diphytanoy-*sn* -glycero-3-phosphocholine (DPhPC)	Avanti Polar Lipids	850356P/850356C
Agarose	Sigma-Aldrich	A9539
Agarose (0.5g Agarose Tablets)	Benchmark	A2501
Alamethicin	AG Scientific	A-1286
Analytical balance	Mettler Toledo	ME204TE/00
Axopatch 200B Amplifier	Molecular Devices	-
BK Precision 4017B 10 MHz DDs Sweep/Function	Digi-Key	BK4017B-ND
Borosilicate Glass Capillaries	World Precision Instruments	1B100F-4
Brain Total Lipid Extracts (Porcine)	Avanti Polar Lipids	131101
DigiData 1440A system	Molecular Devices	-
Extruder Set With Holder/Heating Block	Avanti Polar Lipids	610000
Freezer (-20 °C)	VWR International	SCUCBI0420AD
Glassware	VWR International	-
Hexadecane, 99%	Sigma-Aldrich	544-76-3
Isopropyl Alcohol	VWR International	BDH1133-4LP
Microelectrode Holder	World Precision Instruments	MEH1S
MOPS	Sigma-Aldrich	M1254
Nitrogen (N_2) Gas	Airgas	UN1066
Parafilm M All-Purpose Laboratory Film	Parafilm	PM999
Powder Free Soft Nitrile Examination Gloves	VWR International	CA89-38-272
Precleaned Microscope Sildes	Fisher Scientific	22-267-013
Refrigirator (4 °C)	VWR International	SCUCFS-0504G
Silver wire	GoodFellow	147-346-94
Sodium Chloride (KCl)	Sigma-Aldrich	P3911
Stirring Hot Plate	Thermo Scientific	SP131325
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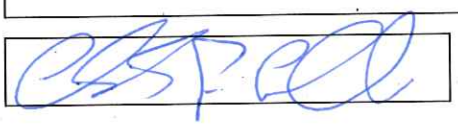
Signature:	
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Dear Dr. Wu,

We would like to thank you for the prompt review of our manuscript. We have made minor changes to the manuscript (tracked) in response to the suggestions. Please see below our point by point responses.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   - We have thoroughly reviewed the manuscript and ensure that the spelling and grammar are correct.

2. Please do not highlight notes for filming.
   - Done.

3. The highlighted protocol steps are over the 2.75 page limit. Please highlight fewer steps for filming.
   - We have removed a couple of protocol steps. We should be within the limit now.

4. The Summary is over the 50 word limit.
   - Done.