Protocol

Preparation of the planar lipid bilayer with following transmembrane protein insertion

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Aim.

To prepare a lipid bilayer suitable for a channel recording, insert a transmembrane protein and obtain channel recordings.

Instrumentation.

The set up consists of an amplifier (Axopatch 200B integrating patch clamp), filter (900 tunable active filter, Frequency Devices Inc.,), heating/cooling bath temperature controller (HCC-100 A), and a sweep function generator (4040 A 20 MHz, BK Precision). The headstage, battery driver magnetic stirrer, and a chamber are located inside the Faraday Cage. The Faraday Cage shields the bilayer form the external static electrical field. The Faraday Cage is placed on top of the vibration isolation table, which reduces the noise and amplifies mechanical stability of the bilayer. Headstage is the heart of the set up and it functions as a current/voltage (I/V) converter. Two Ag-AgCl (immersed in agarose bridges) electrodes connect the chamber with the headstage. The *cis* chamber is grounded and the *trans* chamber is connected to the headstage amplifier (CV 203 BV Axon Instruments).

Method.

The following outline divides the method section into three parts. Part (I) describes the detailed preparation of all solutions used for bilayer formation. It also includes a section on the electrodes preparation. Part (II) provides step-by-step protocol on planar lipid bilayer preparation. The last section (III) outlines the procedure for protein addition after a stable bilayer is obtained.

I. Solutions should be prepared fresh and stored either in a refrigerator (1M KCl, 3M KCl) or in a -20 C freezer (10% (v/v) hexadecane and lipid solution) prior to experiment. Fresh electrodes should be prepared the day before to allow overnight soaking of Ag tips in bleach.

To prepare 50 ml of 1M KCl buffered with 10 mM phosphate (pH 7.4):

a. Prepare 0.1M (7.4 pH) potassium phosphate (PP) buffer: add 19 ml of solution A to 81 ml of solution B, mix and dilute to 200 ml with double distilled H_2O. Use dilution equation (C1V1=C2V2) to prepare 0.01 M potassium phosphate buffer. (For example, take 100 ml of 0.1M (7.4 pH) phosphate buffer and dilute it up to 1 L with a dd water to make 1 L of 0.01 M (10mM) PP buffer.)
b. To make 1 M KCl: weight out 3.7276 g of KCl and transfer it into a 50 ml polystyrene graduate conical tube
c. Dilute to the 50 ml mark with 10 mM (7.4 pH) phosphate buffer.
d. Filter the KCl-PP buffer solution with 2 ul nylon filter.

Alternatively, you may want to prepare a larger volume of KCl and store it in the fridge. Also, you m ay need to make salts of different molarities (e.i 0.5 M or 0.2 M KCl). For example, you may want to make 1 L of 1 M KCl-PP. In this case, weigh out 74.551 g of KCl and transfer it into 1 L volumetric flask. Dilute to the 1 L mark with 10 mM PP buffer.

To prepare 10% (v/v) hexadecane:

a. Using a glass pipette, measure out 900 uL(0.9ml) of pentane (99.9+%, CAS: 109-66-0) and place it into a 2 ml vial (with a screw cap).
b. Using a 100 uL syringe extract 0.1 ml (100 uL) of high purity hexadecane (anhydrous, 99+%, CAS: 544-76-3) and place it into the vial with the pentane.
c. With a glass Pasteur pipette, transfer aliquots of hexadecane into a few 2 ml vials. Place the screw cap on and label with the yellow tape. Tightly wrap with parafilm and store in the -20 C freezer.

To prepare a lipid solution:

a. Obtain a vial with phospholipids (Avanti Polar Lipids, 25 mg 850356 P 1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine) from the -20 freezer
b. Break open the glass top and (using a disposable pipette) add 2.5 ml of high purity pentane into the lipid vial, slightly swirl.
c. Using a glass Pasteur pipette, transfer aliquots of lipid solution into a few (~5) 2 ml glass vials. Place the screw cap on and label with the clear tape. Tightly wrap with parafilm and store in the -20 C freezer.

To prepare Agarose solution:

a. Use 1 gram of Agarose (Bio Rad, # 161-3102) for every 100 ml of solution (e.i 0.2 g per 20 ml; 0.3 g per 30 ml etc…)
b. Place a plastic weighting boat on the scale and tare the scale. Weigh 0.2 g of agarose and transfer into 20 ml volumetric flask.
c. Dilute to the mark with double distilled (dd) H_2O. Transfer the solution into small Erlenmeyer flask.
d. Heat the agarose solution in a microwave for 10-15 seconds (or until solution bubbles). Heated solution is then used to prepare new electrodes.

To make electrodes:

a. Cut ~ 15 cm piece of black PVC hook-up wire (0.21 sq MM). Strip insulation from both ends of wire.
b. Cut ~ 1 cm of silver (1.5 mm diam., CAS 7440-22-4) and carefully wrap one end of PVC wire around a piece of silver.
c. Place a crimp male contact (SPC technology, CCD-201-1-SP) on another end. Place heat-shrink tubing (SR-350 Semi-rigid polyolefin) on both ends and heat over Bunsen burner for a few seconds to secure the connection.
d. Place silver side into a small beaker with Clorox bleach and keep it overnight to coat the Ag.
e. The next morning, remove wire from bleach and rinse with dd H2O, dry with Kimwipe. Using a plastic transfer pipette, fill a pipette tip (SureFit Ergo-F Pipet Tips 10-200 ul) with heated Agarose solution. Insert silver side into the filled pipette cone and top it up with some Agarose solution. Place an assembled construction into a beaker with dd H_2O. When ready to use, cut a tip of a cone with wire cutters and store electrodes in 3 M KCl solution.

To prepare 3M KCl:

a. Electrode tips should be stored in 3 M KCl solution.
b. Weight out 11.1828 g of KCl and transfer it into a 50 ml polystyrene graduate conical tube.
c. Dilute to the 50 ml mark with 0.01 M (7.4 pH) phosphate buffer.
d. Filter via 2 ul nylon filter attached to the 50 ml plastic syringe.

II. To prepare planar lipid bilayer:

1) Rinse the chamber with double distilled H_2O. Then (in the hood) rinse with 95% Ethanol. Dry under the Nitrogen-stream for a minute, carefully moving the drying pipette, but not touching the Teflon walls. Test for dryness. If liquid is visible on the surface- dry for another 20 seconds or so. Be sure to tightly close the Nitrogen gas cylinder. Let the chamber stand for additional 5 minutes to ensure that all ethanol has evaporated.

2) Obtain the vials with lipid solution (clear tape) and hexadecane (yellow tape) from the -20 C freezer. Place the vials into a bucket filled with ice.

3) Obtain a 1M KCl solution.

4) Using a micropipette, add 750 uL of 1 M KCl into both cis and trance chambers through the electrode passages to make sure that they are filled and in contact with *cis* and *trans* chambers (the salt used for solution depends on the experiment).

5) To pre-treat an aperture:

   A 25 um thick Teflon portions the two chambers (*cis* and *trans*). Using a capillary pipette labeled with yellow tape (10 ul pipette) add a full capillary (~10 ul) of 10% (v/v) hexadecane onto the Teflon walls (painting both sides of the Teflon wall) and make sure hexadecane drips all the way down. Hexadecane is crucial for successful bilayer formation and much care should be taken to carefully 'paint' the Teflon walls.

6) To add a lipid solution:

   The lipids should be added right after hexadecane is successfully added onto the Teflon walls. Using a capillary pipette labeled with clear tape (10 ul pipette) add a full capillary (~10 ul) of lipid solution into both *cis* and *trans* chambers. A good way to deliver lipid solution is to submerge a pipette tip into KCl solution. Let the chamber stand for 3-4 minutes to allow hexadecane to evaporate.

7) Place the chamber into the Faraday Cage into the chamber holder. Ground the *cis* chamber and attach electrodes into appropriate electrode cavities inside noise reducing box and chamber. The *cis* chamber is grounded and *trans* chamber is connected to the head stage amplifier. The transmembrane potential is applied through Ag/AgCl electrodes connected to the bath with 3M KCl through agarose bridges.

8) To form a bilayer:

9) A planar bilayer is assembled by bringing two monolayers (one from each chamber) together. Slowly add 750 uL of 1 M KCl to both *cis* and *trans* chambers

through the small pipette holes—this will raise lipids to the aperture and allow the formation of lipid bilayer. The chamber is specifically designed for this volume. Monitor on the amplifier the current readout. Press the 'play' button on the Clampex interface; a straight line at or around 0 pA should be observed. When the bilayer is formed no ions should pass through the bilayer and thus the current should be nearly 0. When the bilayer is broken, the current reading is approximately -18 or -19 nA.

Flip the 'external command' switch on to check the 'quality' of the bilayer. A suitable bilayer will be at (or above) +/- 100 pA. A square function will be displayed on a monitor, with straight lines around 100 pA (a triangular wave is applied, when derivative is taken than displayed function becomes a series of positive and negative straight lines)

A 'perfect' bilayer is around +/- 130 pA. (A word of caution: sometimes you may get a 'bilayer' which is above 150 and even as high as 200 pA. This is NOT necessarily good and may be due to a range of factors: the size of aperture, 'leaky' chamber etc. Similarly, any bilayer below 100 pA should not be typically used for channel recordings.)

Applying voltage to the bilayer will either stabilize or brake the bilayer. After the bilayer capacitance is noted, turn the 'external command' off. A bilayer should be checked for stability by applying positive and negative voltages to it (+/-40 mV and then +/-100 mV). (Don't forget to turn voltage off after testing the bilayer).

III. Adding transmembrane protein (such as alpha-hemolysin) to a stable planar bilayer:

The amount of protein to be added depends on both protein's type and purification protocol. Protein is sensitive to temperature changes and any excessive changes should be avoided. Aliquots of protein should be transferred to labeled small vials and kept at either -80 or -20 freezer (check with someone who may know how to store a specific protein you are working with.) As a general rule, vial containing stock protein should not be taken out. Instead, one should make aliquots of the protein solution (also knows as a 'working' solutions) and keep only as much as needed for the experiment.

1) Transfer needed amount of protein into a small plastic tube and place it into ice bucket. Wait until the protein will thaw out in the ice bucket.

2) Obtain a clean dry stir bar and place it inside the chamber (the same side protein will be added to, but usually *cis* side).

3) Turn the knob to the V hold/I hold position and adjust the voltage command knob to the desired voltage (but keep voltage at 'off' position for now).

4) Add needed amount of protein to the *cis* chamber and turn on the stirrer.

5) Switch the voltage command to the '+' position. By doing so, the voltage will be applied to the membrane. Keep the voltage on.

6) Press play and monitor the current readout. Some proteins (such as *a*-HL) have a well categorized At this point turn off the stirrer to reduce noise.

7) Record for 10 minutes. After recording is complete, hit 'play' button on the screen, switch voltage command to 'off', and then to '-'. By doing so the negative voltage will be applied to the membrane (to check the voltage applied turn the knob to V hold/I hold position).

8) Start recording, by pressing the appropriate button in the Clampex program.

9) Record for 10 minutes. After recording is complete, hit 'play' button on the screen, switch voltage command to 'off' and adjust the voltage to 60 mV. Repeat steps 4-5.

10) Obtain recordings for -60, +80, -80, +100, -100 mV voltages.

References.

Menestrina Gianfranco, Serra Mauro Dalla. Characterization of Molecular Properties of Pore-Forming Toxins with Planar Lipid Bilayers. Methods in Molec. Biology, vol. 145 (171-189).