Protocols

1 PREPARATION OF LIPID VESICLES 2

2 PREPARATION OF MEMBRANE EXTRACTS 3

2.1 Method A 3
2.2 Method B (basic protocol) 4

3 PREPARING LIPID AND MEMBRANE SENSOR SURFACES 4

3.1 Using L1 and HPA chips with lipids and membranes (general protocol) 4
3.2 Cell membrane immobilization on L1 chip 5
3.3 What to expect when using L1 and HPA chips 6
3.4 Cell membrane immobilization on CM5 chip 6
3.5 Control surfaces 6
3.6 Deposition of ligands into a lipid layer 6
3.7 Regeneration and Stability of the Surface 7

4 WORKING WITH RHODOPSIN (CASE STUDY) 8

4.1 Proteoliposome preparation 8
4.2 Proteoliposome immobilization on L1 chip 8
4.3 Proteoliposome immobilization on CM5 chip 9
4.4 On-Surface reconstitution of rhodopsin 9
4.5 Preparation of mixed micelles and optimization of their composition 9

1 Preparation of lipid vesicles

The following references contain useful information regarding the preparation of lipid vesicles:

- *Preparation of Liposomes*, Avanti Polar Lipids, Inc. Product Catalogue; Edition VI; pages 170-172. (Available on-line at http://www.avantilipids.com.)

- New, R.C. *Liposomes: a practical approach*; 1st ed.; Oxford University Press: Oxford, 1990.

1. Dissolve phospholipids in ethanol free chloroform to approximately 10mg/ml.

2. Add lipids at desired composition to glass corex tubes or round bottom flask and deposit as a thin film by removal of the solvent under reduced pressure on a rotary evaporator. Then dry under high vacuum for 2 hours.

   Lipids must be hydrated at temperatures above their phase transition temperature (T_m).

3. Gently resuspend the dried lipid in 120 mM phosphate buffered saline, pH 7.4 (or other buffer with similar ionic strength and pH), to give a final lipid concentration of 20mM. During mixing, it is important not to generate bubbles.

4. Shake lipid suspension for 30 minutes and then sonicate in a bath sonicator for 2 minutes.

   Hydration time may differ slightly among lipid species; however, a hydration time of 1 hour with vigorous shaking, mixing, or stirring is highly recommended.

5. To obtain small unilamellar vesicles, pass the lipid suspension at least 15 times through a 50 nm polycarbonate filter using an extrusion apparatus (available from Avestin or Avanti Polar Lipids, Inc.) to give a translucent solution.

6. Alternatively, small unilamellar vesicles may be formed by sonicating the lipid suspension on ice under a stream of nitrogen, using a direct probe or bath sonicator. For example, perform 10 pulses of 20 s duration at 6µ or 200 W amplitude using a 3 mm microtip probe. The sample should be kept cool with a beaker of ice water and the probe should be ca. 0.5 cm below the surface of the lipid suspension. It is important to wait 20 s between pulses to allow the sample to cool.

   Ultracentrifugation is essential in order to remove titanium particles (from the probe) and to sediment residual multilamellar vesicles leaving a homogenous preparation of small unilamellar vesicles.

   Purify the lipid suspension by ultracentrifugation. Centrifuge the sample for 30 minutes at 15°C at 100,000 g (e.g., 72,000 rpm on a Beckman TL-100

ultracentrifuge). Purified small unilamellar vesicles with a mean diameter of 25 nm will be in the upper layer of the suspension. Transfer the lipid layer, using a Pasteur pipet, to a new tube and store at 4°C under N_2(g).

2 Preparation of membrane extracts

2.1 Method A

1. Harvest cells with standard EDTA or Trypsin solution. EDTA is preferred because no additional protein is introduced. For cells in suspension, harvest by spinning down at 5,000 g for 20 minutes.
2. Resuspend cells in PBS or Tris buffer containing appropriate stabilizers and centrifuge at 5,000 g for 20 minutes.

   Note: pellets can be frozen at this point if necessary.

3. Resuspend pellet in a hypotonic lysis buffer (e.g. 10mM Tris/HCl pH 7.4, 0.5 mM PMSF, and 0.01 mg/ml aprotinin). Use 4 ml lysis buffer per ml of pooled cells.
4. Rupture cells by freeze thawing 4-5 times using a dry ice-ethanol bath and a 37-42°C water bath. Alternatively, the cells may be homogenized.
5. Spin the homogenate at 7,000 g for 15 minutes to remove intact cells and cellular debris.
6. Pellet membranes by spinning supernatant at 200,000 g for 90 minutes.
7. If working with native membranes, then resuspend the pellet in buffer containing 0.5 mM PMSF and 0.01 mg/ml aprotinin.
8. If detergent solubilization of membranes is necessary, proceed by resuspending the pellet in an appropriate detergent and proceed with this protocol.

   Procedures that employ detergents vary depending upon the type of detergent; however, some general steps are applicable to all detergent solubilization procedures.

   a) Solubilize lipids by adding detergent of choice (such as cholate or octylglucoside).

   b) Remove the detergent by dilution, gel filtration, or dialysis. As the detergent concentration decreases, the lipids adopt unilamellar vesicular structures.

9. A final high speed spin (greater than 100,000 g) of the membrane prep supernatant will pellet the microsomal fraction.
10. Resuspend the membrane pellet in buffer. Vortexing or homogenization may be necessary to completely resuspend the pellet. Then, perform a protein determination, aliquot the membrane suspensions and store at -80°C.

2.2 Method B (basic protocol)

1. Wash the adherent cells 3 x with PBS.
2. Put on ice.
3. Cover the cells with a minimum volume of 10 mM Tris pH 7.2
4. Incubate for 30 minutes.
5. Scrape off the cells.
6. Add protease inhibitors if needed.
7. Homogenize with 25 - 30 strokes in a Dounce homogenizer.
8. Centrifuge at 3,000 g for 5 minutes at 4° C.
9. Centrifuge supernatant at 100,000 g for 60 minutes at 4° C.
10. Suspend the pellets in maintenance buffer, of preference at a final concentration of ~10 mg protein/ml. If not used immediately, freeze pellets at -70° C before or after resuspension.

3 Preparing lipid and membrane sensor surfaces

3.1 Using L1 and HPA chips with lipids and membranes (general protocol)

- Lipid layers are best formed at temperatures above the phase transition temperature, T_m of the lipid bilayer. At 25°C, egg yolk PC, DMPC, and unsaturated lipids such as POPC should result in a fluid bilayer.
- Ensure the instrument is clean and detergent-free (i.e. perform a DESORB routine on the instrument and PRIME at least twice with water or detergent-free buffer before docking the L1 or HPA chip).
- Running buffers should be filtered and rigorously degassed daily.

1. Clean the sensor chip with a detergent solution. For the L1 chip use 100 µl of 20 mM CHAPS at a flow rate 10 µl/min. For the HPA chip, use 100 µl of 40 mM octyl D-glucoside at a flow rate of 10 µl/min.
2. Clean injection needle by pre-dipping in water.
3. Immediately inject SUV prep (20 µl, 500 µM) or membrane fractions at a flowrate of 2 µl/min. (If working with membranes, briefly bath sonicate just prior to injection to break up any aggregates).
4. Wash lipid layer at 100 µl/min with NaOH (20mM, 20 µl) and condition surface with regeneration buffer (if regeneration conditions are known). Do

not use NaOH if working with labile natural lipids.

5. Inject BSA (0.1 mg/ml) for 5 minutes to check for non-specific binding (BSA binding should be around 100 RU or less). Caesin (0.1 mg/ml) can be used as an alternative to BSA.

3.2 Cell membrane immobilization on L1 chip

1. Store the membrane vesicles at ~10 mg protein/ml in a stabilizing buffer.
2. Dilute the membranes to 0.1 mg protein/ml with 10 mM acetate, pH 4.0. (a higher pH may be used if the preparation deteriorates). If diluted in a physiological buffer, use 1 - 1.5 mg protein/ml. Add protease inhibitors if needed.
3. Use detergent-free running buffer (e.g. HBS-N). Inject 20 mM CHAPS for 60 seconds at least twice.
4. Inject the membranes at 2 - 5 μl/min until desired level or equilibrium is reached (~1 h).
5. Condition the sensor chip surface with the regeneration method of choice and check the stability.

3.3 What to expect when using L1 and HPA chips

- For the HPA chip, expect a stable signal of between 1500-2000 RU.
- The amount loaded on the L1 chip depends on the type of lipid used. Lipids from native sources (egg, bovine, bacteria etc.) immobilize an intact vesicle layer at 6000-10,000 RU depending on vesicle size (See reference number 6 in the Reference List Section). Smaller vesicles pack more tightly on the surface and give higher loadings. Synthetic lipids form a supported lipid bilayer giving 4000-6000 RU (See reference number 11 in the Reference List Section).
- Lipid membranes should be stable to short exposures of high salt, acid, and base (e.g. 2M NaCl, 10 mM HCl, 10 mM NaOH). **Remember to consider the stability of the ligand when choosing regeneration conditions.**
- Degree of non-specific binding of BSA (especially to the HPA chip) will increase with repeated cycles of lipid loading and cleaning with detergent. If more than 100 RU of BSA binds to the surface following a 5 min. injection at 0.1 mg/ml the flow cell should not be used further.

3.4 Cell membrane immobilization on CM5 chip

1. Store the membrane vesicles at ~10 mg protein/ml in a stabilizing buffer.
2. Dilute the membranes to 0.1 mg protein /ml with 10 mM acetate pH 4.0 - 4.5. Add protease inhibitors if needed.
3. Run the amine coupling wizard. Inject membranes for 30 minutes at 5 μL/min.

4. Condition the surface with the regeneration method of choice and check the stability.

3.5 Control surfaces

- As a general rule, **always** place the control surface flow cell in flow cell 1 to prevent contamination from other flow cells.

3.6 Deposition of ligands into a lipid layer

- Small acylated ligands (<1000 Da) can be inserted directly into a lipid monolayer by injection of dilute solutions (approximately 50 μM) across the monolayer at a flow rate of 10 μl/min.

- Larger molecules (proteins) tend not to associate with a pre-formed lipid layer. Deposition can be achieved by shaking purified protein (100 nM) for 5 minutes with vesicles formed by extrusion (approximately 500 μL) in phosphate buffer followed by loading the surface with the protein containing vesicles. Integral membrane proteins should be incorporated into proteoliposomes by detergent dialysis prior to injection (see Section 4.1).

3.7 Regeneration and Stability of the Surface

- Analytes may be dissociated with variations in pH and ionic strength, leaving the lipid layer intact for additional cycles of interaction.

- The following are reagents that will not destabilize most lipid layers when injected for 2 minutes at a flow rate of 20 μl/min.

    - DMSO, 10%
    - Ethanolamine, 10%
    - Ethanol, 10%
    - NaOH, 10 mM
    - HCl, 10 mM
    - $NaCO_3$, 10 mM
    - Glycine, pH2.0, 10 mM
    - KCl, 2M
    - NaCl, 2M

- Detergents and organic solvents will alter or destabilize membranes or liposomes. A solution of 40 mM octyl D-glucoside may be used to strip the lipids from the L1 or HPA chips in preparation for binding new membranes or liposomes. A solution of 20 mM CHAPS can be used with the L1 chip.

- Natural lipids should not be exposed to NaOH due to possible modification of the head groups.

4 Working with rhodopsin (case study)

4.1 Proteoliposome preparation

1. Evaporate 50 µl egg-phosphatidylcholine (20 mg/ml in chloroform) under a stream of gaseous N_2 and dry for at least 2 h under reduced pressure.

2. Add 1 ml 10 mM Na-phosphate, pH 7.0; 0.15 M NaCl; 1 mM $MgCl_2$ and 50 mM octylglucoside. Shake occasionally over a period of 30 minutes or continue until the lipids are solubilized.

3. Mix 14 µl 60 µM rhodopsin with 190 µl of the lipid solution. Dilute to 0.5 ml with 10 mM Na-phosphate, pH 7.0; 0.15 M NaCl; 1 mM $MgCl_2$ and 50 mM octylglucoside. This gives final concentrations of 0.5 mM lipid and 1.7 µM rhodopsin.

4. Dilute a further 190 µl of the lipid preparation to 0.5 ml as reference liposomes.

5. Dialyze the preparations (membrane poresize limit 6000 Da) against 1 liter 10 mM Na-phosphate, pH 7.0; 0.15 M NaCl and 1 mM $MgCl_2$, at 4° C. Change the buffer after 4 h and let stand over night.

6. Transfer the preparations to Eppendorf tubes and centrifuge for 5 - 10 minutes to remove any precipitates. Store preparations on ice.

- All steps involving the use of rhodopsin must be performed in darkness or under safelight.

4.2 Proteoliposome immobilization on L1 chip

1. Use detergent free running buffer (e.g. HBS-N). Inject 20 mM CHAPS for 60 seconds at least twice

2. Inject the proteoliposomes at 2 µl/min until desired level or equilibrium is reached (~1 h). The proteoliposomes can be diluted 2-5-fold with 10 mM acetate, pH 4.0 in order to increase the efficiency of immobilization.

3. Condition the surface with the regeneration method of choice and check the stability.

4.3 Proteoliposome immobilization on CM5 chip

1. Dilute the proteoliposomes 2-5-fold with 10 mM acetate, pH 4.0.

2. Run the amine coupling wizard. Inject proteoliposomes for 15 minutes at 5 µl/minute.

3. Condition the surface with the regeneration method of choice and check the stability.

4.4 On-Surface reconstitution of rhodopsin

1. Wash the Pioneer Chip L1 with two injections of 20 mM CHAPS for 1 minute.
2. Activate the surface with an injection of EDC/NHS for 7 minutes.
3. Inject rhodopsin at 0.6 μM in 10 mM maleate, pH 6.0 and 20 mM octylglucoside for at least 14 minutes.
4. Block the chip surface with 0.96 M ethanolamine-HCl, pH 8.5 and 20 mM octylglucoside for 7 minutes.
5. Immediately reconstitute the immobilized rhodopsin with a 1 - 2 minute injection of mixed micelles (3.3 mM POPC and 25 mM octylglucoside in HBS-N).

4.5 Preparation of mixed micelles and optimization of their composition

1. Pipette a 10 mM solution of lipids in chloroform into round bottomed glass tubes, pre-washed in chloroform, to give a final concentration between 0.1 and 10 mM.
2. Evaporate under a stream of nitrogen gas. Remove residual solvent under reduced pressure for at least 2 hours.
3. Dilute octylglucoside from a 0.5 M stock to 20 - 40 mM with HBS-N to yield 10 mM HEPES, pH 7.4 and 150 mM NaCl (HBS-OG).
4. Add HBS-OG to the dry lipid film and shake the mixtures every 10 minutes for at least 45 minutes. Check turbidity by eye.
5. Condition the L1-surface with five 30 second injections of 20 mM CHAPS.
6. Inject the mixed micelles for 1 - 8 minutes at 5 μl/minute.
7. Regenerate the sensor surface with two 1 minute injections of 50 mM octylglucoside.

- It is important to vary the concentrations of both the lipid and the detergent during optimization. Five different concentrations of each, in the ranges given above or close to the optima, are recommended.