Bovine Outer Segment Isolation

(Protocol based off of, and with grateful acknowledgement to, Papermaster, Methods Enzymol. v. 81, p. 48 (1982)., Mao Y and Finnemann SC. Methods Mol. Biol. v. 935, p. 285 (2013)., and Parinot C, et al. JoVE. doi.org/10.3791/52100 (2014).)

- Note, one bovine eye yields approximately the same number of outer segments as three porcine eyes, and this protocol can be adjusted accordingly for porcine eyes.
- The protocol below is scaled for 75 bovine eyes. Adjust volumes based on whether eyes are porcine or bovine, and how many eyes will be dissected.

Materials:

Solutions

1) 200mM Tris/Acetate pH 7.2 - dissolve 12.1g Tris base in 400mL deionized water (ddH_20); adjust pH to 7.2 with glacial acetic acid; fill up to 500mL in graduated cylinder with ddH_20. Sterile filter.

2) 1M $MgCl_2$ solution - dissolve 100g $MgCl_2*6H_20$ into ddH_20 up to a total volume of 490mL.

3) 40mM phosphate buffer, pH 7.2 (2x solution) - mix 1.88g of NaH_2PO_4 (anhydrous) with 6.54g of $Na_2HPO_4*7H_20$ into 950mL of ddH_20. Adjust pH to 7.2 with 2N NaOH (just a few drops to start with). Add additional ddH_20 until volume is 1L.

Prepare the following working solutions fresh on the day of the photoreceptor outer segment (POS) isolation using the stock solutions. Solution concentrations and volumes are critical since density of the solutions matters.

1) 100mM glucose (10x) stock for the day - dissolve 900mg of glucose in ddH_20, fill to 50mL.

2) Taurine 50mM (10x) stock for the day - dissolve 626mg of taurine in ddH_20, fill to 100mL.

3) 55.36% w/v sucrose stock solution (300mL) – dissolve 166.08g of sucrose into ddH_20 to a total volume of 250mL. Stir at RT for 15-30 min until fully dissolved. After sucrose is dissolved, add ddH_20 such that final volume of solution is EXACTLY 300mL. To ensure that the volume of the final solution is accurate, weigh the beaker used to make this solution and use this as the zeroed weight. A 55.36% sucrose solution should have a density of 1.2596g/mL, so a solution that is exactly 300mL should have a net weight of 377.88mg.

4) Homogenization solution (90mL) - 20% w/v sucrose (32.5mL of 55.36% sucrose stock solution), 9 mL of 200mM Tris/Acetate stock (10x), 180µL of 1M $MgCl_2$ stock (500x), 9mL of 100mM glucose stock (10x), 9mL of 50mM taurine stock (10x), and 30.31mL of ddH20.

5) Sucrose gradient mixtures – prepare 90mL of each of the following solutions:

* 28.5% w/v sucrose solution (90mL) – mix 46.33mL of 55.36% sucrose stock solution with 16.67mL ddH_20 and 9mL of 50mM taurine stock (10x) and 9mL of 200 mM Tris/Acetate stock (10x) and 9mL of 100 mM glucose stock (10x). Syringe filter.

* 34% w/v sucrose solution (90mL) – mix 55.27mL of 55.36% sucrose stock solution with 7.73mL ddH_20 and 9mL of 50mM taurine stock (10x) and 9mL of 200mM Tris/Acetate stock (10x) and 9mL of 100mM glucose stock (10x). Syringe filter.

* 38.75% w/v sucrose solution (90mL) – mix 63mL of 55.36% sucrose stock solution with 9mL of 50mM taurine stock (10x) and 9mL of 200 mM Tris/Acetate stock (10x) and 9mL of 100mM glucose stock (10x). Syringe filter.

6) WASH 1 (420mL) – mix 42mL of 200mM Tris/Acetate stock (10x) with 42mL of 50mM taurine stock (10x) with 336mL of ddH20. Syringe filter.

7) WASH 2 (90mL) – 10% w/v sucrose (16.26mL of 55.36% sucrose stock solution), 9mL of 200mM Tris/Acetate stock (10x), 9mL of 50mM taurine stock (10x), 55.74mL of ddH20. Syringe filter.

8) WASH 3 (30mL) - 10% sucrose w/v sucrose (5.42mL of 55.36% sucrose stock solution), 15mL of 40mM phosphate buffer stock (2x), 3mL of 50mM taurine stock (10x), 6.58mL of ddH20. Syringe filter.

9) Storage solution (175mL) – 2.5% w/v sucrose (7.9mL of 55.36% sucrose stock solution) combined with 167.1mL of DMEM/F12 (any brand will work – if you only have alpha-MEM, should be ok too). Syringe filter.

<u>Other Materials</u>

Absorbent pads to cover surfaces.

Razor blade or surgical blade. Inoculating loop. Iris scissors. Metal spatula. Bunsen burner for sterilization.

EasyStrainer 100µm cell strainers (ideally, ~20) – Greiner Bio-One catalogue #542000

Ultracentrifuge tubes (~ 10) with 38.5 mL max capacity – Beckman Coulter catalogue #326823

Ultracentrifuge such as Beckman Optima L-90K with rotor such as Sorvall SureSpin 630 swinging bucket

Centrifuge that can accommodate 15mL and 50mL Falcon Corning conical tubes

Long spinal needle and 10mL syringe for creating sucrose gradients – EXELint Medical Products catalogue #26960 and BD Biosciences catalogue #309604

18-guage needle and 10mL syringe for obtaining outer segment band from centrifuged sucrose gradients – EXELint Medical Products catalogue #26419 and BD Biosciences catalogue #309604

Dark box containing a large beaker filled with ice to keep tubes with collected retinas

Ice buckets with sheet to cover and protect from light

Microcentrifuge tubes, Falcon 15mL and 50mL conical tubes

P1000 and P200 pipettes with tips and scissor to cut off pipette tip ends

Hemocytometer

Ice-cold iodine solution

Ice-cold sterile PBS

If ordering dark-adapted already-isolated bovine retinas, place order with WL Lawson Company (wllawsoncompany.com/). Otherwise, obtain 75 bovine retinas from local slaughterhouse.

Protocol:

1) Note that unlike most outer segment isolation protocols, this one is NOT done in the dark. However, it is still worthwhile keeping the eyes and retinas away from as much light as possible.
2) Prechill all solutions on ice. *Keep all materials ice-cold at all times unless otherwise specified in this protocol*. Place ultracentrifuge rotor and swinging buckets at 4°C.
3) Obtain 75 bovine eyes from a slaughterhouse and process them as fresh as possible. Do not use frozen eyes. Chill eyes in a balanced salt solution and keep them in the dark. If obtaining pre-dissected, dark-adapted retinas shipped unfrozen from WL Lawson Company, skip steps 3-6.
4) Trim away excess connective tissue, then incubate in ice-cold iodine solution for 5-10 min, followed by 2x rinse in sterile PBS. Keep eyes not currently being dissected in ice-cold PBS in a cooler free from light.
5) Dissect eyes under as dim an ambient light as possible without compromising visualization. Take one eye firmly into non-dominant hand. Cut approximately 2-3mm behind limbus and remove anterior segment. Flip the eyecup inside out. Use spatula to gently dislodge retina, pushing the retina towards the optic nerve.
6) Collect 25 bovine retinas in a 50mL Falcon conical tube containing 15mL homogenization solution on ice. Start a new Falcon tube for each set of 25 bovine retinas.
7) If obtaining retinas from WL Lawson Company, place each set of 25 bovine retinas into a 50mL conical tube with 15mL of homogenization solution.
8) Rock each tube containing retina and homogenization solution gently for 2 min (vigorous shaking will fragment outer segments). Filter through the cell strainer, using a sterile p1000 pipette tip to move the fatty solution over the strainer mesh to keep the flow going. When the first strainer clogs even with using a pipette tip, use a new strainer to filter remaining homogenization solution in the Falcon tube.
9) Prepare sucrose gradients – each ultracentrifuge tube can hold approximately 8 bovine retinas or ~1/3 of the homogenization solution with outer segments from a single 50mL Falcon conical tube. The ultracentrifuge has a capacity of ~35mL. Using a 10mL syringe and spinal needle, place 9mL of the 28.5% sucrose solution on bottom of tube. Next, carefully inject 10mL of the 34% sucrose

solution at the base of the tube, ensuring that the line between the two sucrose densities is preserved. Next, inject 6mL of the 38.75% sucrose solution at the base of the tube. Be careful when inserting and removing the needle to avoid causing a disruption/mixing at the sucrose density interfaces. When entering the tube with a full syringe, be careful not to allow drops of the higher density sucrose to escape the needle until it is at the bottom of the ultracentrifuge tube.

10) Overlay 5-9mL of homogenization solution onto the top of each density gradient. Balance tubes to within 0.1g by using homogenization solution.
11) Spin immediately in the ultracentrifuge using a swinging bucket rotor (Sorvall Surespin 630, 25k rpm, 48 min, 4°C).
12) With outer segments from fresh retinas harvested in the dark, there is usually a single red to orange to pink band at the 28.5%/34% sucrose interface. As this band is held up to the light, it will photobleach to a brown color. When you have a very large outer segment prep, you can see some red to orange to pink banding at the other interfaces, but they should be much less prominent than the primary band. We do not collect any outer segments from these other interfaces, as they are substantially contaminated by other particles. Outer segments from frozen retinas or light-exposed retinas may demonstrate some banding at the 34%/38.75% sucrose interface. A small number of organelles may remain at this interface as well.
13) The 28.5%/34% sucrose interface may be recovered readily by aspirating the homogenization solution and the homogenization/28.5% sucrose interface with a serologic pipette. Leave as much of the 28.5% sucrose layer as possible. Next, holding the ultracentrifuge tube against a solid vertical surface, puncture the tube wall at a level just beneath the 28.5%/34% sucrose interface with an 18-gauge needle attached to a 10cc syringe. Prior to puncturing, release the 10cc syringe plunger so that it can be easily drawn back. Collect as much of the 28.5%/34% sucrose interface as possible without collecting anything turbid at the homogenization/28.5% sucrose interface or the 34%/38.75% sucrose interface.
14) After removing the needle from the ultracentrifuge tube, draw the solution up from the needle and entirely into the syringe before removing the needle from the syringe and gently pushing the solution into a 50mL Falcon conical tube. Dilute with 5 volumes of ice-cold WASH 1.
15) After collecting all outer segments at the 28.5%/34% sucrose interface (which may require more than one round of ultracentrifugation for a large prep size like 75 bovine retinas), spin each conical tube containing the WASH1-diluted outer segments at 4°C, 3,000 × *g* for 10 min.
16) Gently resuspend each pellet in 10 mL ice-cold WASH 2; minimize pipetting and flick tube as much as possible. Combine resuspended solutions into the minimum number of 50mL Falcon conical tubes necessary and spin again at 4°C, 3,000 × *g* for 10 min.
17) Gently resuspend each pellet with ice-cold WASH 3, splitting the 30mL of total WASH 3 solution evenly between the conical tubes. Minimize pipetting in favor of flicking tube as much as possible. Combine all resuspended solutions and split them into two 50mL Falcon conical tubes, each with 15mL of WASH3-outer segment solution. Spin again at 4°C, 3,000 × *g* for 10 min.
18) If outer segments are to be labeled with a fluorescent dye, resuspend the outer segments in labeling buffer (see separate protocol). A minimum of $2x10^8$ outer segments should be used for each labeling procedure. If not labeling, resuspend the purified outer segments from 75 bovine retinas in 36mL of the storage solution.

    Dilute 10µL of the storage solution-outer segment mixture with 490µL of pure storage solution (1:50) in a microcentrifuge tube and mix well by pipetting. Count diluted outer segments in a hemocytometer to calculate yield and concentration. Multiply the number seen in the large square

containing 4x4 smaller squares (upper and lower corners) by the dilution factor of 50 and then by the hemocytometer adjustment factor (usually 10,000x). This yields a concentration in units of OS/mL. OS should look like small rods and circles. They should not look like black sand particles (this indicates excessive shearing forces during the prep).

19) Dilute to ~1 × 10^8 OS/mL with storage solution. Distribute into 500µL or 1mL aliquots, snapfreeze with liquid nitrogen, and store at −80°C. Avoid freeze-thaws.

Yields

When using bovine eyes from WL Lawson Company, the yield from 75 bovine retinas is typically 1 – 1.5x10^{10} outer segments