Twist-off hair-bundle isolation using agarose

Peter Gillespie
last modified 95/09/25

Cell-Tak coverslip preparation

(1) It is recommended to prepare 10-20 ring/coverslips at a time. Clean coverslip/rings (coverslip with a nylon washer cemented on with fingernail polish) by soaking with 20% H_2SO_4 for 30 minutes. Rinse several times with water.

(2) Treat the coverslip/ring with 1 N NaOH in ethanol (ethanolic KOH) for 30 minutes. Rinse very thoroughly with ethanol, then with water.

(3) Soak with 1 mg/ml poly-L-lysine for 60 minutes. Rinse with water. The ring/coverslips can be dried and stored at room temperature indefinitely.

Cell-Tak coating

(4) IMPORTANT: the Cell-Tak coating procedure needs to be initiated **at least 2 hours before you expect to use it**.

(5) Spread on 10 μl of 1.8 mg/ml Cell-Tak, being absolutely sure not to let the drop of fluid touch the ring (it won't dry properly if it touches). Place on the 65°C hot block and dry for 30 minutes.

(6) Apply three 5-μl coats, spreading across the entire surface each time. Before the Cell-Tak fully dries, it is useful to spread the fluid again. It is best to have the last coat finish drying within 30 minutes of use. Rinse with SSPI before use.

Preparation of SSPI

(7) Make up fresh 0.7 μg/ml pepstatin in ethanol if tube in freezer is > 2 weeks old. Make up fresh 0.2 M PMSF in ethanol (sonicate to get into solution).

(8) Thaw three 10 ml aliquots of 10x standard saline and one tube of 0.5 mg/ml leupeptin. Mix 270 ml water, 30 ml 10x standard saline, and 0.3 ml aliquots of the three protease inhibitors. Use promptly as the PMSF has a half-life of 0.5-1 hour in this solution.

Other pre-dissection chores

(9) Pour 100 ml SSPI into a 100-ml Eerlenmeyer and pump it through the superfusion system so that it is ready to go. Replace the 0.45 μm in-line filter if necessary.

(10) Fill another 100-ml Erlenmeyer with SSPI, then fill two 35-mm plastic petrie dishes with about 5 ml each of SSPI.

(11) Load a 20 ml syringe with SSPI and replace the 0.45 μm filter if necessary. Squirt 10 ml of SSPI into a 60-mm glass petrie dish in the 34°C water bath; place a petrie dish lid over the dish (with the lid upside down so no scummy water is wicked into the clean SSPI). Place two other empty petrie dishes into the water bath. Make sure that the water bath comes up about 1/2 way on the petrie dishes.

(12) Add 0.75 g of agarose to a tube and fill to 25 ml (3% agarose). Microwave at medium power for 35 seconds (or until solubilized), then store in the 65°C water bath until needed.

(13) Prepare labeling reagents, if necessary. Get frogs and body bag. Weigh microfuge tubes for twist-offs, if necessary. Assemble tools and get scalpel blades.

Dissections

(14) Dissect 1-16 sacculae, using SSPI. It is best to cut the endolymphatic sac as closely as possible to the actual edge of the sacculus. The nerve should also be cut as close as possible to the back side, beveled if possible. The strands on the back side of the sacculus should be cut as closely as possible. The goal of the dissection is to leave as flat a surface as possible; this aids the adhesion to the Cell-Tak-coated coverslip. Save all of the ears in a covered petrie dish until all dissections are done.

Sticking the ears down

(15) Transfer the ears to another petrie dish that has a Cell-tak-coated ring/coverslip.

(16) Using coarse forceps, stick the sacculi to the coverslip. (Two pairs of forceps can be handy.) Use a gentle hand so that the ears are not damaged too much. Cut edges may contribute substantial amounts of contaminants. The ears should lie as flat as possible on the coverslip. Pack them together well so that many can be fit on a single coverslip (maximum 20). I have found no consistent relationship between position on the coverslip and yield. Leave at least one large gap (even better two at 180° from each other) for the blunt needle stub that is used to twist the agarose gel.

Other pre-agarose steps

(17) Carefully remove the otolithic membranes. I use fine forceps, coming down at a 45° angle, so that I don't actually hit very many bundles.

(18) If not labeling the bundles prior to isolation, put the agarose in the 34°C water bath at this time. Remove the glass thermometer from the water bath and carefully clean it with ethanol, drying thoroughly. Stick it into the 3% agarose tube.

(19) Gently wash the dish with several changes of filtered SSPI, aspirating in between washes. For some of the washing, put the syringe needle on one side of the washer and the aspirator needle on the other, and direct a stream across the ears. Make sure there is always sufficient buffer in the chamber formed by the washer.

(20) The ears can be labeled at this stage with sulfo-NHS-biotin, sulfo-SHPP, or NHS-biotin at this stage. Afterwards, the ears are washed with 1 mM lysine in SSPI (3x) then SSPI.

Twist-offs

(21) Using the coarse curved forceps, take the coverslip with ears out of its petrie dish and place it into a 60 mm glass petrie dish in the 34°C water bath. Agitate the coverslip a little to make sure that warm buffer exchanges into the well formed by the nylon washer. Incubate 3-4 seconds.

(22) Aspirate away the solution (except that retained by the nylon washer). Using a P5000, squirt agarose directly on the ears, displacing saline. Repeat the careful pouring action with the 3% agarose.

(23) Quickly, before the agarose hardens, pull the coverslip out of the petrie dish with agarose in it (with the curved forceps) and transfer to an empty petrie dish. Tip the dish 30-45° briefly to get the level of the agarose at about the washer height. Incubate at 34°C for 1 minute. Do not agitate.

(24) Place in 4°C refrigerator for 10 minutes to allow the agarose to solidify. Cover the dish to keep crap out. You can leave them at 4°C longer than 10 minutes if you need to.

(25) Remove the loose agarose around the outside of the washer using forceps. Move the coverslip out of the dish to the stage under better illumination. Score around the inside of the washer with a blunt tungsten needle. Try to make sure there are **no upwards forces** on the agarose at this stage, as these will make the bundles slip out of the agarose. This scoring step is a critical step.

(26) After the score, plunge a 15-g luer stub needle (orange flange) all the way through the agarose to the coverslip at one of the gaps with no ears, then twist with a sharp rotatory motion. This is the twist-off. The degree of twist is not at all important – in principle, a few micrometers is all that is necessary. (Note that you usually need to ream out the 15-g stub with an 18-g stub, as dried agarose is usually jamming the stub you want to use.)

(27) Using forceps, drop the disk of agarose upside-down (bundles up) into a nylon washer on a microscope slide (on the stage).

(28) If any of the ears remained imbedded in the agarose, grasp them with the forceps and give them a sharp tug perpendicular to the bundles (the "jerk-off"). My best technique is to put one tine of the forceps down to the side of the sensory epithelium and push down and away, minimizing the upwards forces that allow the bundles to slip out of the agarose.

(29) Immediately rinse the agarose where the bundles are with filtered SSPI. Start the perfusion stream (filtered SSPI). Perfuse for 10-15 minutes. During this time, I score around all of the sets of bundles with a fine tungsten needle. I also cut away any agarose that is close to bundles and that has a lot of contaminating debris.

(30) Remove the bundles by cutting a chunk of agarose that contains the bundles, spearing it with the tungsten needle, and transfering it to a microfuge tube (preweighed or filled with buffer).

Clean-up chores

(31) Put away all of the tools and return the microscope to its normal position. Make sure that the dissecting area and the area around the sink are cleaner than you found them.

(32) Run 50-100 ml of water through the perfusion system, then run air through. This is important, as otherwise bugs will grow in the lines, contaminating the preps. I sometimes run through some 1 N HCl, followed by a lot of water, to clean the lines. In addition, I often replace the tubing that runs between the filter and the outflow with fresh tubing.