DISSECTION MANUAL
for the
MOUSE TEMPORAL BONE

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INTRODUCTION

The membranous labyrinth containing the organ of Corti is housed within the bony labyrinth of the temporal bone. In order to collect quantitative data (e.g., number of missing hair cells), it is best to examine the organ of Corti as a 'surface' or 'flat' preparation. One technique for making surface preparations involves dissecting the cochlea in liquid, removing segments of the organ of Corti and mounting them on microscope slides (e.g., Engström et al., 1966; Norris et al., 1977; Ohlemiller et al., 1999). After fixation, the cochlea is immersed in buffer or ethanol. The cochlear bone is broken away with hooks and forceps. The spiral ligament, Reissner's membrane and the tectorial membrane are removed from the specimen with small forceps and scissors. Using a small knife, the organ of Corti is divided into short segments which are then carefully separated from the modiolus. The organ-of-Corti segments are then mounted on glass slides in a liquid medium such as glycerin, cover slipped and examined microscopically. The advantage of this 'wet' surface preparation technique is that the organ of Corti can be examined microscopically on the day of specimen fixation. Disadvantages of this technique include: 1) Preparation artifacts such as distortion or loss of sensory and supporting cells often occur in portions of the organ of Corti because the sensory epithelium is dissected while it is immersed in a liquid medium. Quantitative data cannot be collected when segments have been damaged or destroyed; and 2) The soft tissue of the cochlea deteriorate if the specimen is left in buffer or alcohol for more than a few days. Dissected segments of the organ of Corti that are mounted in liquid also deteriorate within a few weeks.

Another way in which to obtain surface preparations of the organ of Corti is to embed the temporal bone in plastic after fixation, then dissect the specimen after the plastic polymerizes. Bohne and Harding (1997) and Bohne et al. (2001) described the steps required for fixing, dehydrating and embedding the mouse temporal bone in plastic. The advantages of this 'plastic-embedded' surface preparation technique are: 1) Dissection artifacts are rare; and 2) Plastic-embedded cochleae and organ-of-Corti segments are stable and can be dissected and evaluated years after fixation and embedding. The disadvantage of this technique is that more time is required between specimen fixation and evaluation. Because of the time required for plastic polymerization, the earliest that a plastic-embedded cochlea can be examined microscopically is approximately six days after specimen fixation.

Preparation for perfusion fixation:

Cover two 30G x1/2" needles with PE-20 tubing, leaving about 2 mm of the tip uncovered. Fill one 12 ml plastic syringe with 6 ml of sterile, lactated Ringer's solution (LRS) and a second 12-ml syringe with 6 ml of fixative [e.g., 1% osmium tetroxide in Dalton's buffer (Appendix A); 4% paraformaldehyde in 0.1 M PO_4 buffer]. Put one of the covered needles on each syringe.

Assemble the instruments (Fig. 1), supplies and equipment needed for the perfusion, including:

1 or more scintillation vials (20 ml) containing 18 ml of fixative stoppered with a cork stopper. These vials are used for filling the fixative perfusion syringe.

10-ml specimen vials - each containing 4-ml of fixative and stoppered with a cork stopper. One vial is needed for each mouse to be sacrificed. Use paper tape and a pencil to

mark the appropriate animal # on each vial.

2" deep dish with ice to keep vials of fixative cold.
Paper tape
Several 4" x 4" gauze sponges
500 ml of Hank's balanced salt solution
500 ml of 70% ethanol
#3 scalpel handle (Storz Instruments)
#10 scalpel blade
Small curved hemostat (Storz E3916)
Medium straight hemostat (Storz E3920)
Medium rat-tooth forceps (Storz E1650)
Small rat-tooth forceps (Fine Science Tools 11066-07)
Medium straight, sharp-tipped scissors (Storz N1410)
Small straight, sharp-tipped scissors (FST 14084-08)
Fine #5 Dumont forceps
Stapes hook (Storz N1698-56)
Diamond-tipped forceps (Dumont #3 with diamonds)
2 sharp-pointed picks mounted in pin vises (Appendix B)
10 fine-tipped glass pipettes (Appendix C), the non-tapered ends wrapped with waterproof tape
Cochlear perfusion apparatus (Appendix G).
Mouse perfusion board
1-ml syringe filled with ml of 0.25% Marcaine
Suction apparatus
2 - FR #3 suction tips
Operating microscope
Dissecting microscope (15-40X)

Anesthesia and surgery for whole body perfusion:

Weigh the mouse. The anesthetic mixture (ketamine-xylazine) should be given intraperitoneally at a dosage of ketamine - 0.08 mg/g and xylazine - 0.15 mg/g body weight.

After anesthetizing the mouse, place it on its back and tape it to the perfusion board (Fig. 2). Inject 0.25 ml of 0.25% Marcaine into the chest wall on either side of the rib cage. Wet the belly fur with water and palpate the xiphoid process. Under the operating microscope, make a small incision transversely at the level of the xiphoid process with the scalpel (Fig. 2). With the medium rat-toothed forceps, elevate the skin rostral to the incision. Use the medium scissors to cut through both sides of the rib cage as far as the front legs. Reflect the chest flap (Fig. 3) toward the neck and clamp with a small hemostat.

Identify the left ventricle and right atrium. With the small scissors, make a small cut in the right atrium. Clamp the abdominal aorta with the medium forceps just rostral to the diaphragm. Use the LRS syringe to puncture the left ventricle of the heart. Insert the needle into the left ventricle near the apex of the heart. The PE tubing on the needle should keep the needle from penetrating entirely through the heart. Slowly inject the solution into the left ventricle while watching the fluid escape from the right atrium. A surgical assistant should

use the suction apparatus to carefully remove the escaping blood, LRS or fixative (see below) from near the right side of the heart and rostral to the diaphragm. Perfusion should continue until the returning fluid is nearly colorless. When the blood has been washed out of the vascular system, remove the LRS syringe and replace with the fixative syringe. Try to place the needle on the fixative syringe into the original hole created by the LRS syringe. If the original hole is used, you are less likely to create a 'short-circuit' for the fixative (i.e., fixative going into and out of the left ventricle). Perfuse 6 ml of fixative through the vascular system. **Be certain to have the assistant suction the escaping fixative from the right side of the heart to avoid inhaling fixative fumes.**

After completing the vascular perfusion, blot the remaining fixative from the chest and abdominal cavity with several 4x4 gauze sponges. Remove the tape from the animal. Hold the animal by its head and flex its neck dorsally. Carefully decapitate the mouse with the sharp scissors while avoiding the jaw. With the scalpel, skin the dorsal, lateral and ventral sides of the head as far as the orbits. On each side, cut through the external auditory meatus. Use the medium scissors to split the dorsal skull, from the foramen magnum to the orbits. Cut the skull laterally, posterior to the orbits, on both sides. Bend the flaps outward to reveal the brain. Carefully remove the brain from the cranial cavity.

Just anterior to the foramen magnum on the floor of the skull, the bony labyrinths will be seen (Fig. 4). Use suction to remove the flocculus from each temporal bone. Using the tips of the diamond-tipped forceps (held together) as a dissection device, cut the fibrous connections between the temporal bone and the rest of the skull. If done correctly, the entire temporal bone, including the bulla, will separate from the skull. If you are not interested in the middle ear, open the bulla, remove the malleus and incus, then locate the cochlea, stapes and round window. Dislocate the stapes from its position in the oval window with the stapes hook. With a sharpened pick, make a small hole in the cochlear bone near the apex of the cochlear duct. Fill one of the glass pipettes with fixative. Slowly perfuse the fixative into the apical hole in the cochlear bone. If the hole is patent, the fluid should escape from the oval window. Turn the temporal bone over so the internal auditory meatus is visible. With the diamond-tipped forceps, make an opening in the superior semicircular canal. Drip fixative into this opening, as well as the cavity that contained the flocculus. When finished, immerse the temporal bone in the vial of fixative for 2 hours (OsO_4) or 4-18 hours for paraformaldehyde.

After the fixation period, rinse the specimens in HBSS three times (i.e., 15 minutes for each change) then immersed the specimens in 70% ethanol. Once they are immersed in ethanol, examine the specimens carefully under a dissection microscope (40X). From each specimen, remove the extraneous soft tissue and facial nerve using the diamond-tipped forceps. Remove the stapes with the stapes hook or the diamond forceps. With a small pick, open an infiltration hole in the cochlear bone at the apex and in scala tympani at the base, just rostral to the internal auditory meatus. Carefully remove debris, bone chips and bubbles from the cochlear scalae with a glass pipette and gentle mouth suction. The trimmed specimens should be transferred to 20-ml scintillation vials (containing 4 ml of 70% ethanol) and remain refrigerated overnight.

Be certain to place all fixative-contaminated material (e.g., gauze sponges, kimwipes, used

pipettes, used fixative and HBSS contaminated with fixative into a toxic waste jar for safe disposal).

Dehydration and embedding schedule

The morning after the cochleae are fixed, immerse them in a dish of 70% ethanol. Under the dissection microscope, check them for bubbles and debris in the fluid spaces (e.g., scalae vestibuli and tympani) before starting dehydration (Fig. 5A). Set the timer (be certain to wind and set the alarm) for each of the steps.

Dehydrate the specimens and infiltrate them with plastic according to the following schedule. **Note that the cochlear bone is not decalcified before embedding.** The specimen vials should be rotated continuously on a bottle rotator in between solution changes:

20 min - 80% ethanol 20 min - 95% ethanol 20 min - 100% ethanol 20 min - 100% ethanol 20 min - propylene oxide 20 min - propylene oxide	**Do not** drain all of the solution from the vial. Leave sufficient solution in vial so cochlea is never left completely dry. Make quick double changes of each vial for all solutions. Pour used propylene oxide into waste araldite-propylene oxide jar.

Make single changes of vial contents for all of the rest of the steps.

20 min - propylene oxide:araldite = 2:1 20 min - propylene oxide:araldite = 1:1 20 min - propylene oxide:araldite = 1:2	Mix in graduated cylinder 20 minutes before needed. Cover cylinder with Parafilm to prevent evaporation of propylene oxide.

1 hour - araldite 1 (bubble check 1 at ½ hr point) 1 hour - araldite 2 (bubble check 2 at ½ hr point) 1 hour - araldite 3 (bubble check 3 at ½ hr point) 1 hour - araldite 4 (bubble check 4 at ½ hr point)	Leave caps off vials & shine 100-W light on vials to drive off residual propylene oxide.

If no time is lost during the solution changes and bubble checks, this schedule requires 7 hours to complete. However, several minutes are usually lost during each bubble check so that it generally takes longer than 7 hours to dehydrate, infiltrate and embed cochleae (Fig. 5B). Embedding mouse cochleae in plastic requires much time and effort to do correctly. Because bubbles prevent fluid exchange between the vial and the cochlear scalae, they must be removed when found. Because of the time spent on solution changes and bubble checks, it is important to sacrifice no more than 6 mice (and embed 12 cochleae) at a given time.

When ready for embedding, the two cochleae (round and oval windows facing up) from an individual mouse should be placed on opposite sides of a medium Peel-away mold (R-30) containing a 3-mm-thick layer of fresh, liquid plastic. The plastic is polymerized for 48 hours at 60° C.

Use a waste jar to dispose of the plastic dilutions and the straight changes of araldite safely.

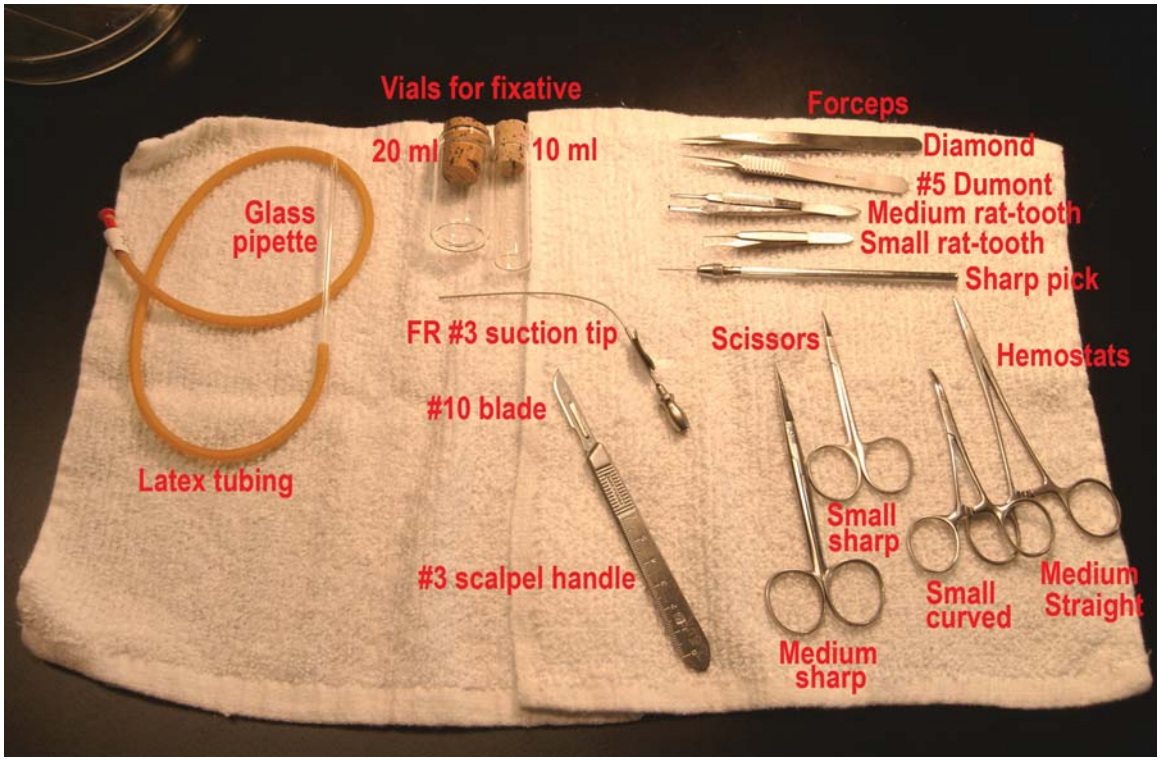


Figure 1: Surgical instruments needed for vascular perfusion of the mouse.

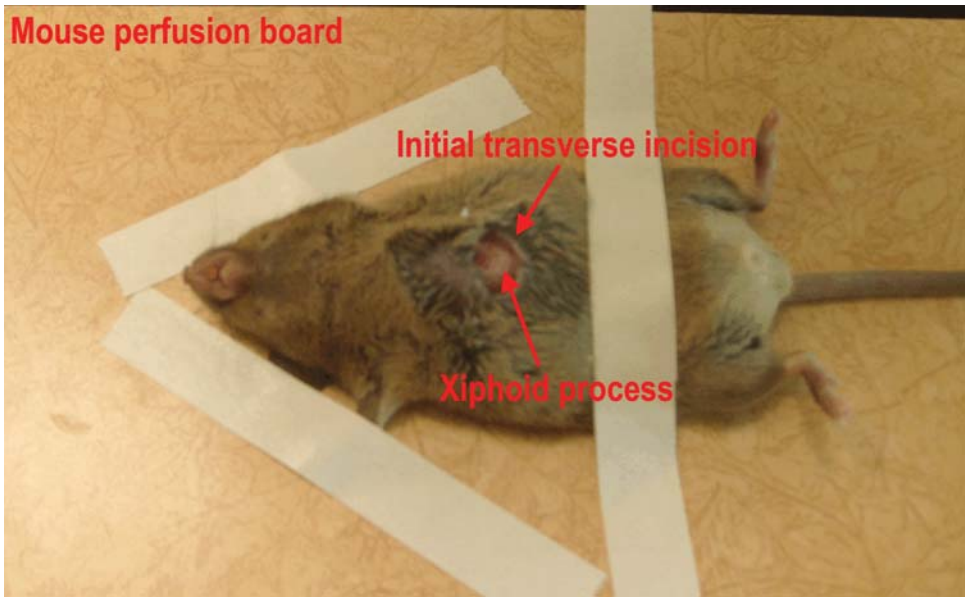


Figure 2: Anesthetized mouse taped to perfusion board. The xiphoid process of sternum appears as a slight protuberance between the two halves of the rib cage. The initial transverse incision has been made across the xiphoid.

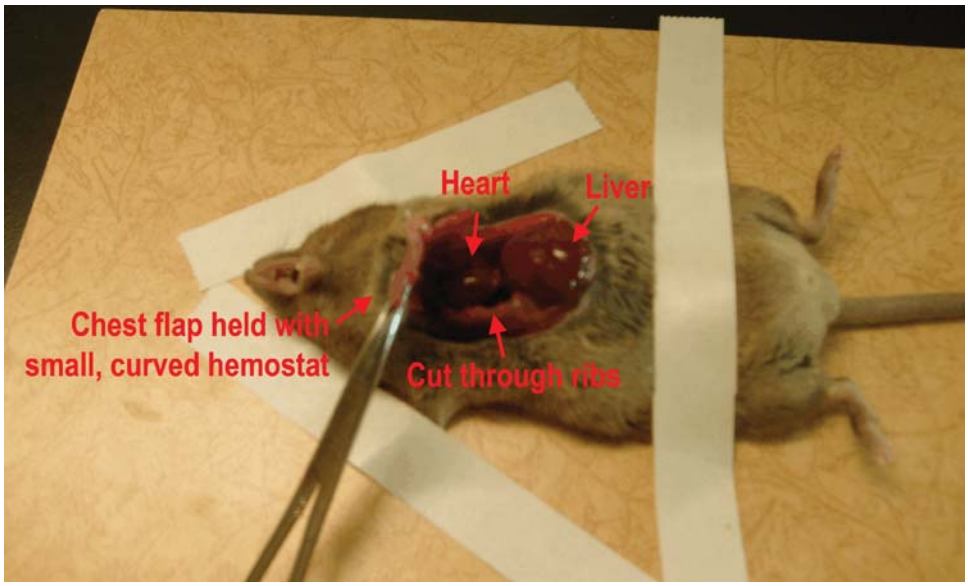
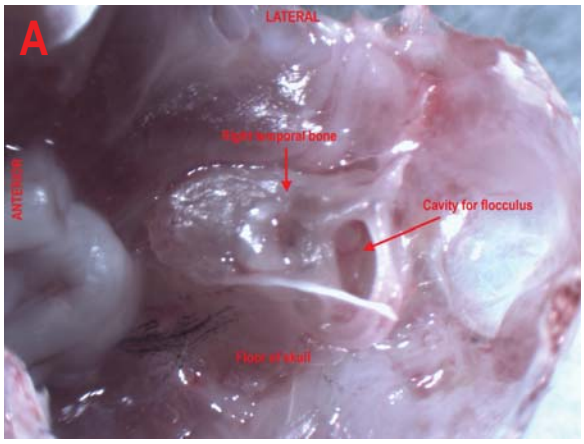


Figure 3: Chest flap has been opened to reveal the heart and liver.



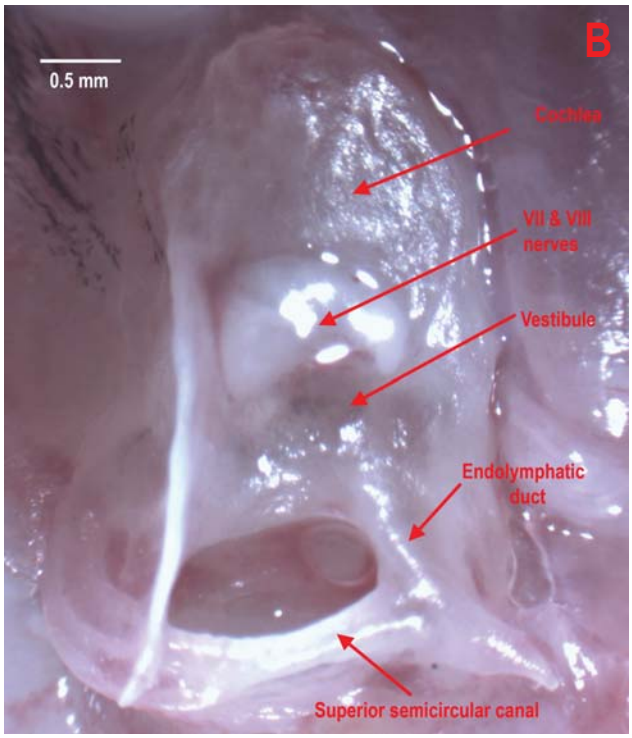


Figure 4: Right temporal bone viewed from inside of the cranial cavity. A) The line around the temporal bone represents the fibrous connection between the temporal bone & skull; B) At a higher magnification, various parts of the inner ear are visible. The flocculus of the cerebellum was removed with the rest of the brain.

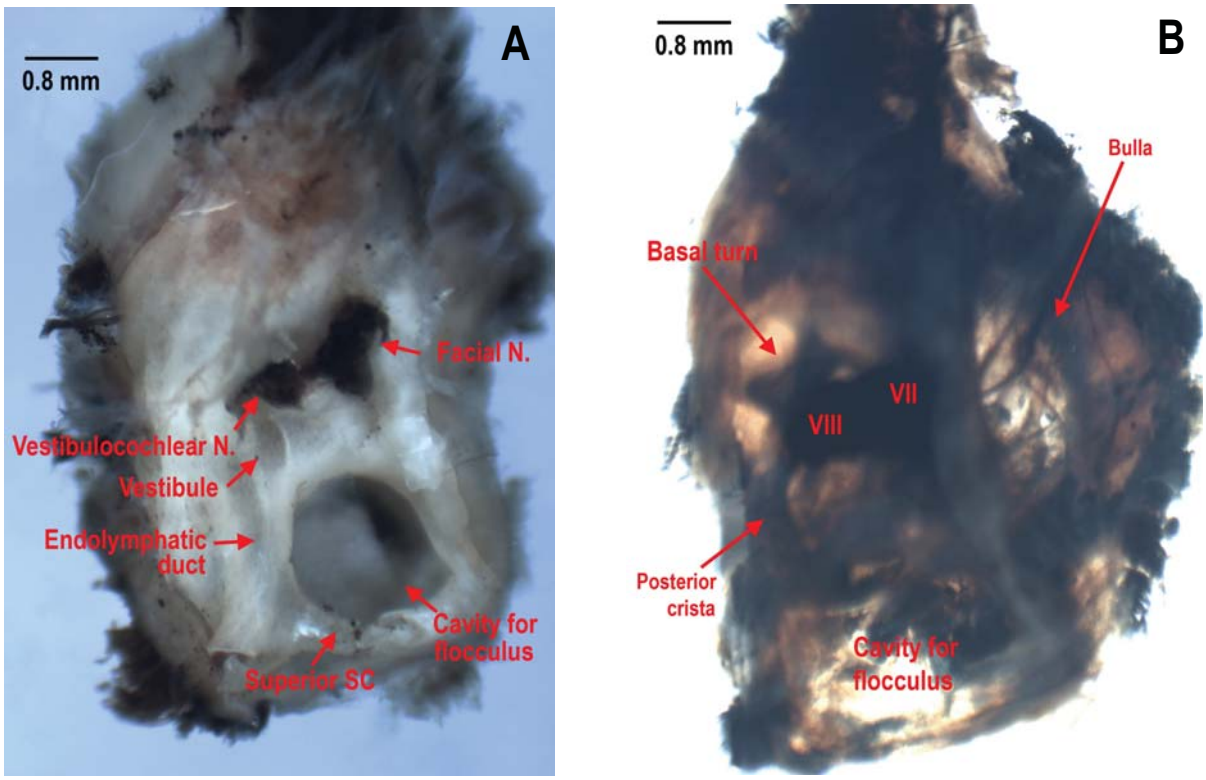


Figure 5: View of the dorsal surface of the mouse's left temporal bone, including: the vestibulocochlear nerve (VIII), facial nerve (VII); superior semicircular canal (SC); posterior crista, cavity for the flocculus and bulla. A) Specimen fixed with OsO_4; B) Specimen fixed with OsO_4, decalcified, dehydrated and infiltrated with plastic.

Preparation of the middle and inner ears for microscopic examination

If it is necessary to examine the middle ear and ossicles for pathological changes, it is advisable to embed and section the entire temporal bone. In this instance, after removing the fixed temporal bone from the skull, trim away the extraneous soft tissue but leave the bulla intact. After washing, immerse the temporal bone in a decalcifying solution (e.g., 0.1 M EDTA in 0.1 M PO_4 buffer or PBS and change daily for 3-4 days; Wang et al, 2002; Meyer zum Gottesberge & Felix, 2005). Dehydrate, then infiltrate and embed the decalcified temporal bone in a support medium like paraffin or plastic. Once the support medium solidifies or polymerizes, cut sections through the entire temporal bone (Fig. 6) using a microtome. Mount the sections on slides, stain, coverslip and examine by bright-field microscopy.

Horizontal sections of mouse middle & inner ears

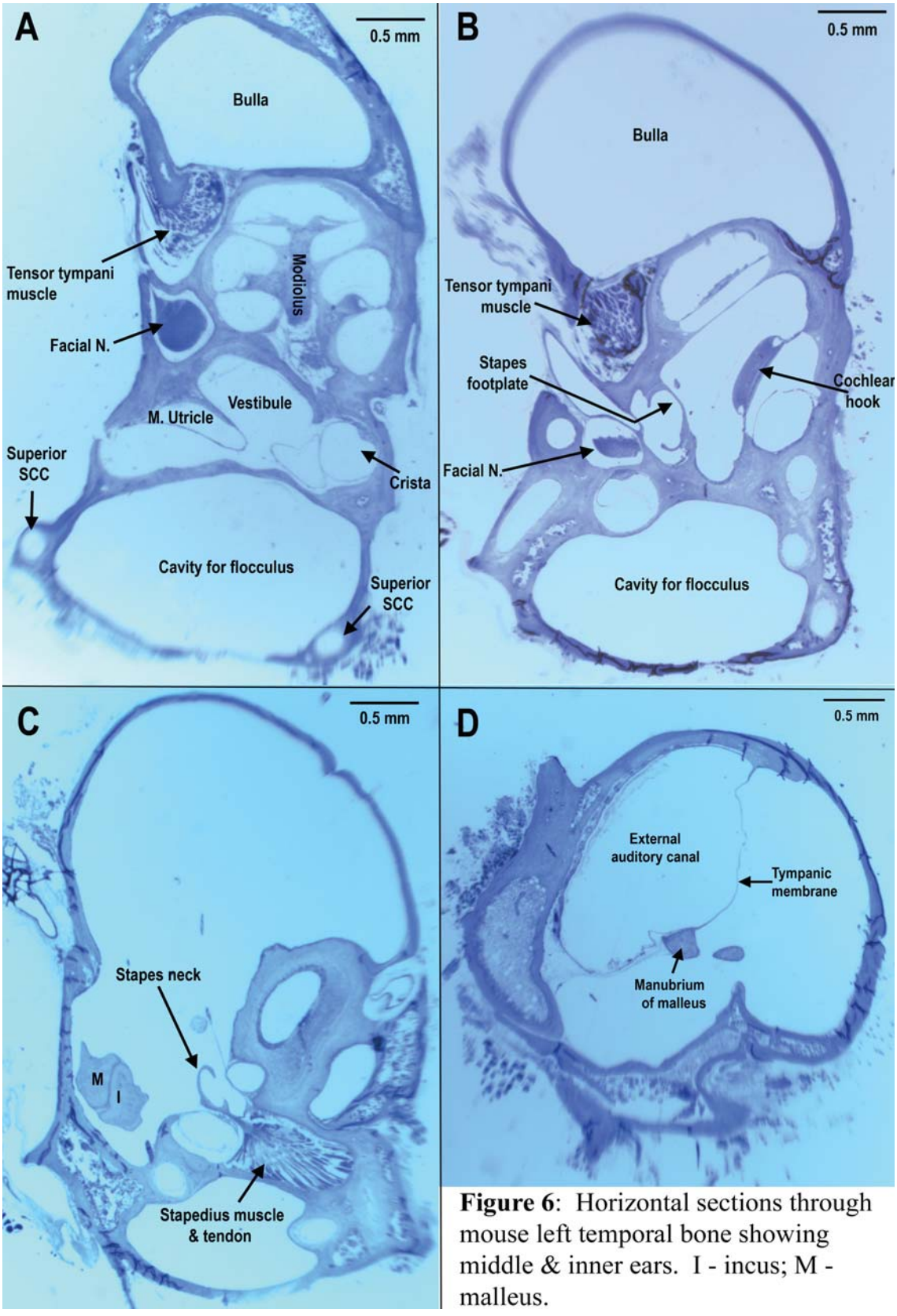


Figure 6: Horizontal sections through mouse left temporal bone showing middle & inner ears. I - incus; M - malleus.

First Dissection of the Cochlea

Remove the araldite block with embedded cochleae from the Peel-away mold. Roughly saw out one cochlea, leaving a 'handle' of plastic below the round and oval windows and semicircular canals. Mount the block in an aluminum specimen chuck so that the entire cochlea (Fig. 7) is visible above the rim of the chuck. At this point, note the position of the apical- and basal-turn infiltration holes, the round and oval windows (Fig. 7) and the interscalar septum (Fig. 8). These features can best be seen through smooth plastic. When the bone is being chipped away later, it will be more difficult to see these structures.

Removal of the araldite outside the cochlea is done at 40X magnification under the dissection microscope using quarter pieces of double-edged razor blades (Appendix E). Because it is important to see the cochlea as clearly as possible, the razor blades should be changed often as their edges become nicked. After removing most of the outside plastic, coat the specimen with a thin layer of liquid plastic. The liquid plastic improves the optical clarity of the specimen. Continue using razor blades to remove thin layers of plastic from the specimen. When the cochlear bone is reached, the araldite will peel away fairly cleanly. Cut slowly and carefully when removing the plastic because the bony labyrinth of the mouse is so thin that it is easy to cut through it with a razor blade.

It is easier to remove the araldite on the sides of the cochlea by turning the specimen chuck on its side in the trimming stand. To stabilize the chuck, hold it tightly between the left forefinger and the left thumb while resting the chuck on the edge of the trimming stand. The chuck can be tipped up or down on the trimming stand to maintain focus so you do not have to constantly refocus the microscope. To prevent part of the cochlea from snapping off, it is important to work around the specimen turn by turn until the round and oval windows are reached. Do not take off araldite below this point so that there will be adequate support for chipping off the cochlear bone. Be certain to remove all plastic outside the cochlear bone so that the next time plastic is encountered (i.e., after bone removal), it will be the plastic filling the cochlear scalae.

Starting at the apex, remove the cochlear bone using a freshly sharpened pick (Appendix B). Both the point of the pick and the beveled edges are used to chip or flake off the undecalcified bone. The cochlear bone is quite thin in the mouse. It takes very little effort to chip the bone away from the plastic-filled cochlear scalae. It is very important to remove the bone from apex to base so as not to crack the cochlea off the handle. In addition, the bone has to be flaked off carefully to avoid chipping off the underlying spiral ligament and stria vascularis.

Once bone removal has been completed (Fig. 9), re-coat the specimen with liquid plastic to wash away any adhering debris and bone chips. Prepare two Peel-away molds (R-40) containing a 1.5-mm-thick layer of liquid plastic and labels for the five half turns of cochlear duct in one boat (i.e., 1-2, 3-4, 5-6, 7-8, 9-10) and labels for the utricular macula, (MU) saccular macula (MS), and cristae of the horizontal (HC), superior (SC) and posterior (PC) canals in the other boat, along with the ear number. (**Note**: Do not use molds impregnated with silicone for any embedding step because the silicone will permeate the plastic and degrade its quality.)

At 40X magnification, carefully observe the liquid plastic-coated specimen and note the spiral of the cochlear duct, the location of the apical and basal tips of the cochlear duct and the location of the nerves to the cristae of the three semicircular canals (Figs. 10 and 11). It is difficult to see the saccular macula at this point and impossible to see the utricular macula.

Quarter pieces of razor blades are used to separate half-turns of the cochlear duct from the remainder of the specimen (Figs. 12 and 13). Starting at the apex, make the first cut perpendicular to the organ of Corti cutting as far as the basilar membrane while avoiding the apical end of the cochlear duct. The first horizontal cut should go through the interscalar septum (Figs. 8 and 9) beneath the apical half-turn. The first perpendicular and horizontal cuts meet at the helicotrema and result in the separation of the apical half-turn (labeled 1-2) from the rest of the specimen. Use diamond-tipped forceps to place segment 1-2 below its label in the Peel-away mold.

Coat the remainder of the specimen with liquid plastic to improve visibility. Identify the cut edge of the cochlear duct in the specimen. The second perpendicular cut should be made a half-turn away from the first perpendicular cut (Figs. 12 and 14). The second horizontal cut should go through the interscalar septum beneath the next half-turn (i.e., segment 3-4). The second perpendicular and horizontal cuts meet at the modiolus and result in the separation of the second half-turn from the rest of the specimen. Put segment 3-4 in the Peel-away mold below its label.

Coat the remainder of the specimen with liquid plastic to improve visibility. Identify the cut edge of the cochlear duct in the specimen. The third perpendicular cut (Figs. 12 and 15) should be made a half-turn away from the second perpendicular cut. The third horizontal cut should go through the interscalar septum beneath the third half-turn. The third perpendicular and horizontal cuts meet at the modiolus and result in the separation of the third half-turn (labeled 5-6) from the rest of the specimen. Put segment 5-6 in the Peel-away mold below its label.

The cut between the lower first turn (segment 7-8) and round window (segment 9-10) should come at the location where the organ of Corti turns and begins its posterior descent. The round window region must be exposed by picking away the bone and cutting away excess araldite. In this area, the cochlear duct follows the lip of the round window.

Once segment 7-8 is removed from the specimen, separate the block containing segment 9-10 and the maculae and cristae (in the vestibule) from the handle by cutting through the superior canal with razor blades. Place this block on the glass plate of the dissection microscope in a pool of liquid araldite. Remove extraneous tissue from the block (e.g., facial nerve; lateral part of oval window; superior and posterior canals) by making careful razor cuts. The saccular macula is located deep to the oval window. The utricular macula is oriented at a right angle to the saccular macula and is caudal to it.

Using a quarter blade, separate the maculae and the lateral and superior cristae from the remainder of the block. Separate the posterior crista from cochlear duct segment 9-10. The posterior crista is deep to the round window. Carefully separate the lateral and superior cristae from the maculae. Finally, separate the saccular and utricular maculae.

The half-turns of cochlear duct should be oriented in the boat so that the cut edges of the organ of Corti face the segment labels and the basilar membrane side of each segment faces the bottom of the mold (Figs. 16 and 17). The separated vestibular organs should be oriented so that the surface of the sensory epithelium is clearly visible through the plastic.

The 1st dissection molds only needs to be polymerized long enough for the plastic to harden so that the individual segments can be sawed out in preparation for the 2nd dissection. Usually, polymerization overnight (i.e., 12-16 hours) at 60° C is sufficient.

Second Dissection of the Cochlear Duct

Take the first dissection boat from the oven and allow it to cool to room temperature before removing the plastic block from the Peel-away mold. Under a dissection microscope, use a fine pair of forceps (tips together) to mark off the approximate locations for sawing out each half-turn segment (Fig. 18). Note that the plastic is cut close to the cut edges of the organ of Corti while a 'handle' of blank plastic is left opposite the cut edges to serve for holding the segment during trimming.

Prepare a Peel-away mold (R-40) with a 1.5-mm-thick layer of liquid plastic with labels for the ten quarter turns of cochlear duct (i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10), as well as the ear number. Use the medium rat-tooth forceps to hold segment 1-2 by its handle under the dissecting microscope with the basilar membrane facing up. Coat the segment with liquid plastic. Use razor blade middles (wedged between the thumb and forefinger) to trim the plastic in scala tympani close (approximately 0.1 mm) and parallel to the basilar membrane. Cut the segment off the handle and divide it in half with a new quarter blade. The apical tip is segment #1 and the other part is segment #2. Use the diamond-tipped forceps to turn segment 1 on edge so the cut edge of the organ of Corti is visible and re-coat with liquid plastic. Note if the plastic has been trimmed close and parallel to the basilar membrane (Fig. 19). If the trimming below the basilar membrane is satisfactory, hold the segment with the diamond forceps so that you can trim away any bone above Reissner's membrane (Fig. 19). When trimming is satisfactory, put segment 1 below its label in the Peel-away mold with the basilar membrane side facing the bottom of the container. Check segment 2 for appropriate trimming then put it in the Peel-away mold.

Continue trimming the other half turns of the cochlear duct in the same fashion. Segment 3-4 will be split into segments 3 and 4. Segment 5-6 will be split into segments 5 and 6. If segment 7-8 is short, do not split it. Otherwise, split segment 7-8 into segments 7 and 8. Finally, split segment 9-10 into segments 9 and 10 (Figs. 20 and 21).

The second dissection mold is polymerized for 48 hours at 60°C. After removing it from the oven, allow the polymerized block to cool to room temperature before removing it from the Peel-away mold. The upper edges of the block can be trimmed flat with a razor blade or ground with a bench grinder. To examine the organ of Corti at a high magnification (i.e., 500-1000x), turn the second-dissection block upside down. Put a small droplet of immersion oil over the segment that is to be examined. Put the segment under an oil immersion objective, immerse the lens in the oil droplet and focus with the coarse and fine focus knobs (Appendix F).

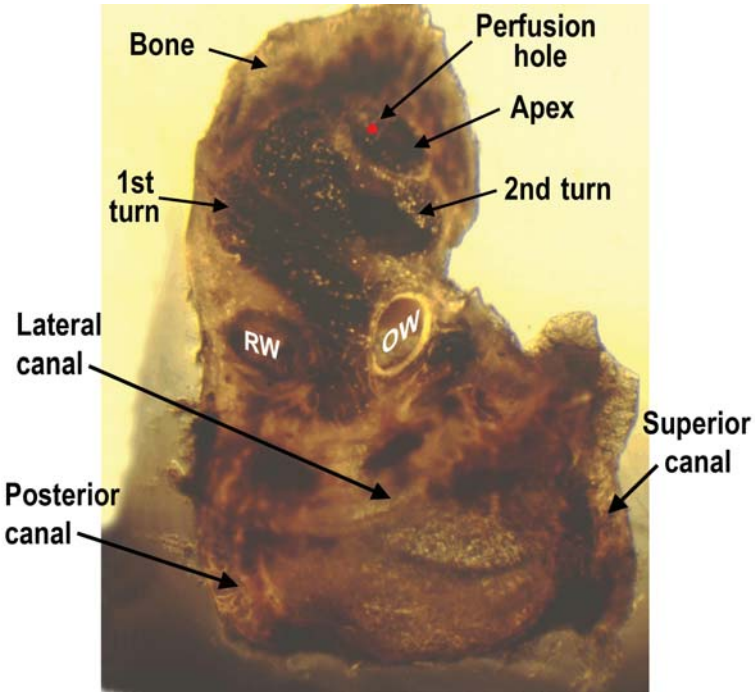


Figure 7: Dissection microscope view of the left cochlea (fixed with OsO_4) as viewed from the ventral side where the round (RW) and oval (OW) windows are visible. The cochlea was dehydrated and infiltrated with plastic while most of the cochlear bone was intact. When the mouse cochlea is dehydrated, the cochlear bone becomes transparent and the membranous labyrinth can be seen in its entirety from basal to the apical tip. The red dot shows the approximate location for the inlet hole in the cochlear bone that is used to perfuse fixative through the perilymphatic spaces. The perfusion outlet is the oval window.

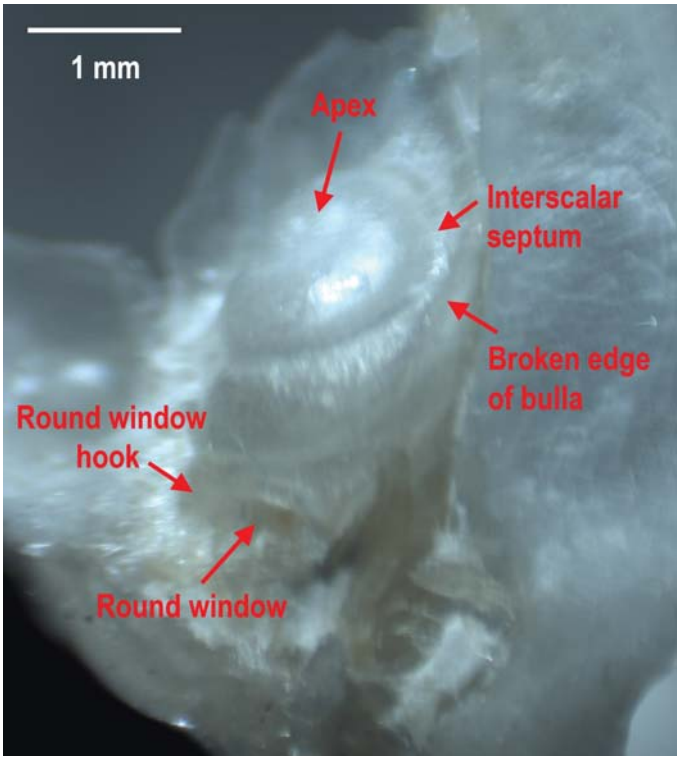


Figure 8: With the bullar wall widely opened, the complete spiral of the right cochlea from apex to round-window hook is visible. In this dried preparation, the location of the interscalar septum, which separates scala tympani of the upper cochlear turn from scala vestibuli of the lower turn, appears as an indentation on the cochlear bone.

MOUSE COCHLEA AFTER REMOVAL OF COCHLEAR BONE

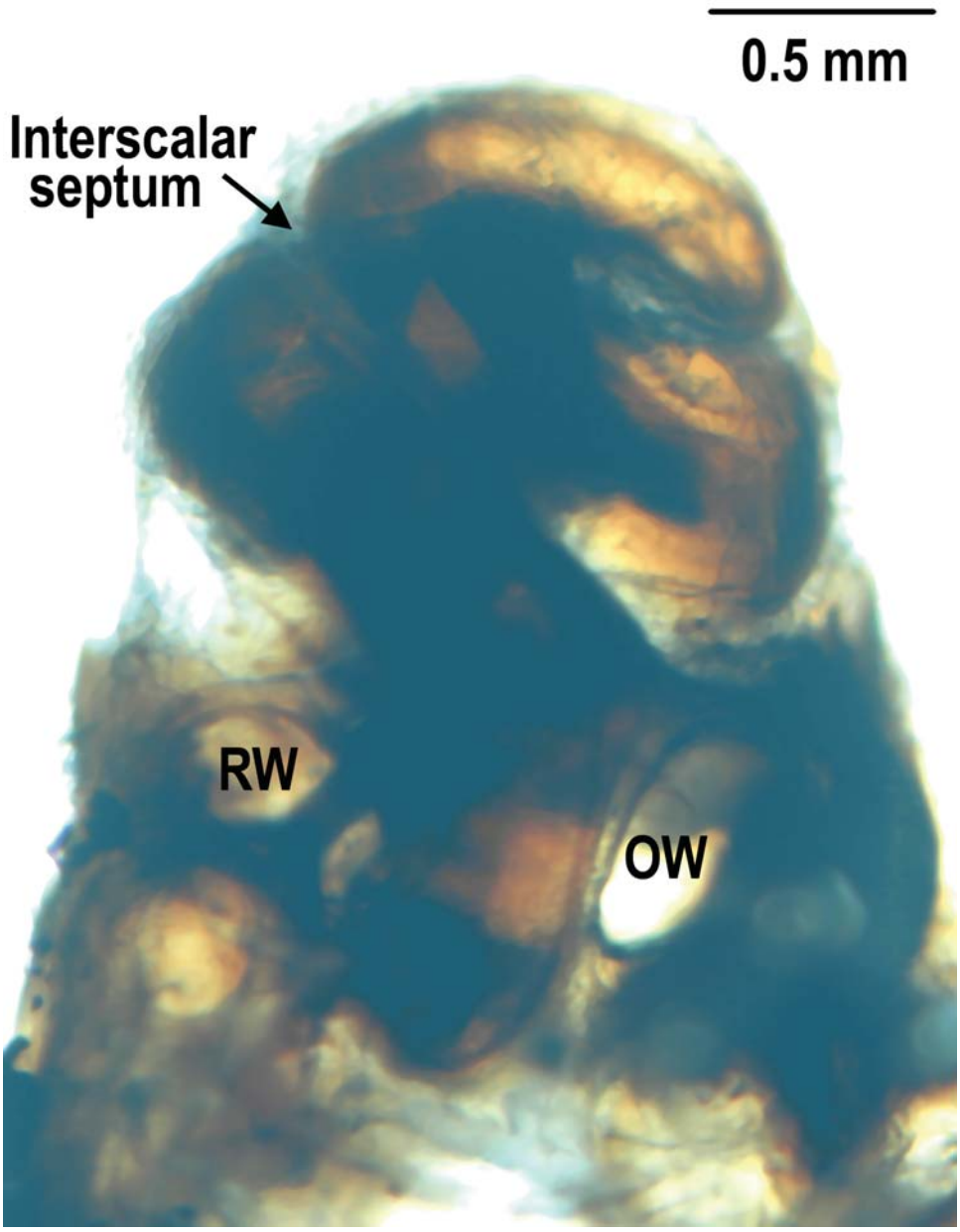


Figure 9: Using a sharpened pick, the cochlear bone is chipped away from the membranous labyrinth from apex to base. When finished, the remaining bone includes the interscalar septum, the modiolus (not visible here) and the rim of the round (RW) and oval (OW) windows. The goal here is to remove sufficient bone to be able to follow the curve of the cochlear duct and to identify the interscalar septum. It is not necessary to remove every bit of cochlear bone.

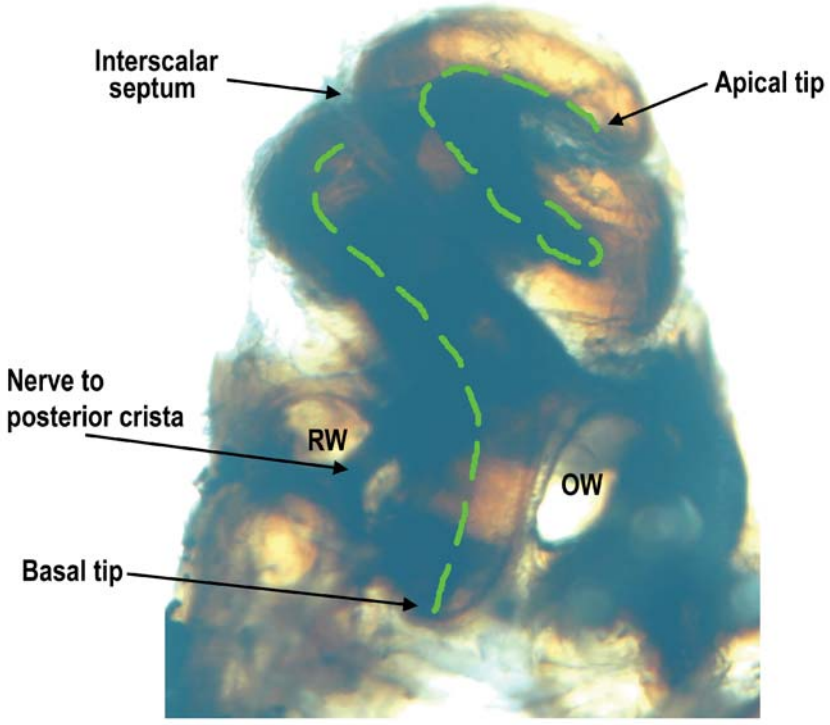


Figure 10: A dashed green line marks the approximate location of the osseous spiral lamina as it spirals from the basal to the apical tip. The nerve to the posterior crista can be seen through the round window.

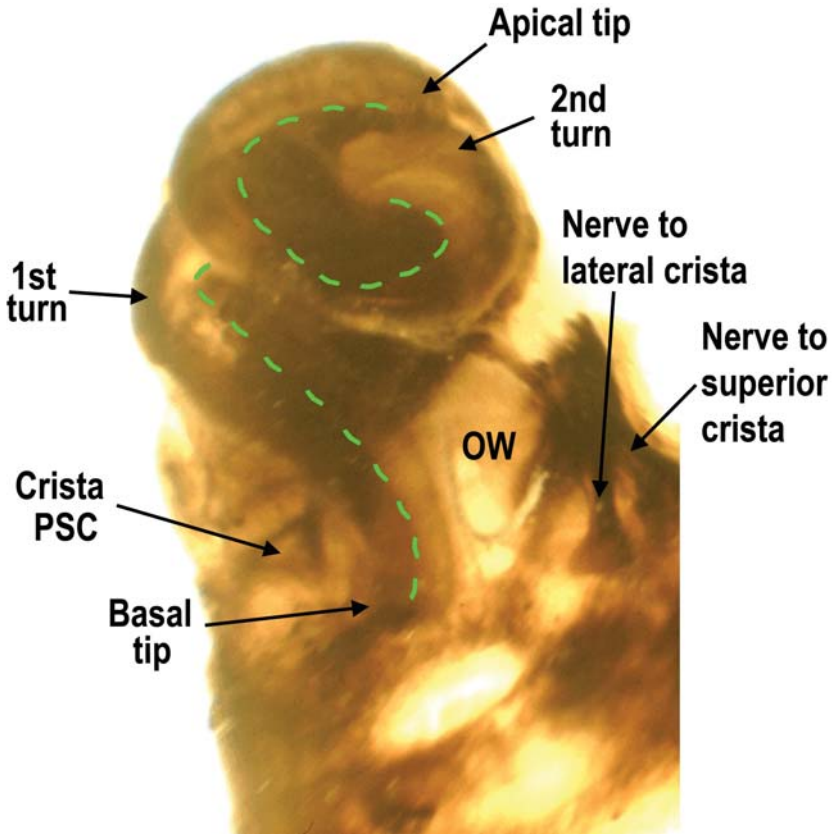


Figure 11: By changing the microscope focus, the nerves to the three semicircular canal cristae can be seen.

DIVISION OF MOUSE COCHLEAR DUCT INTO 5 SEGMENTS

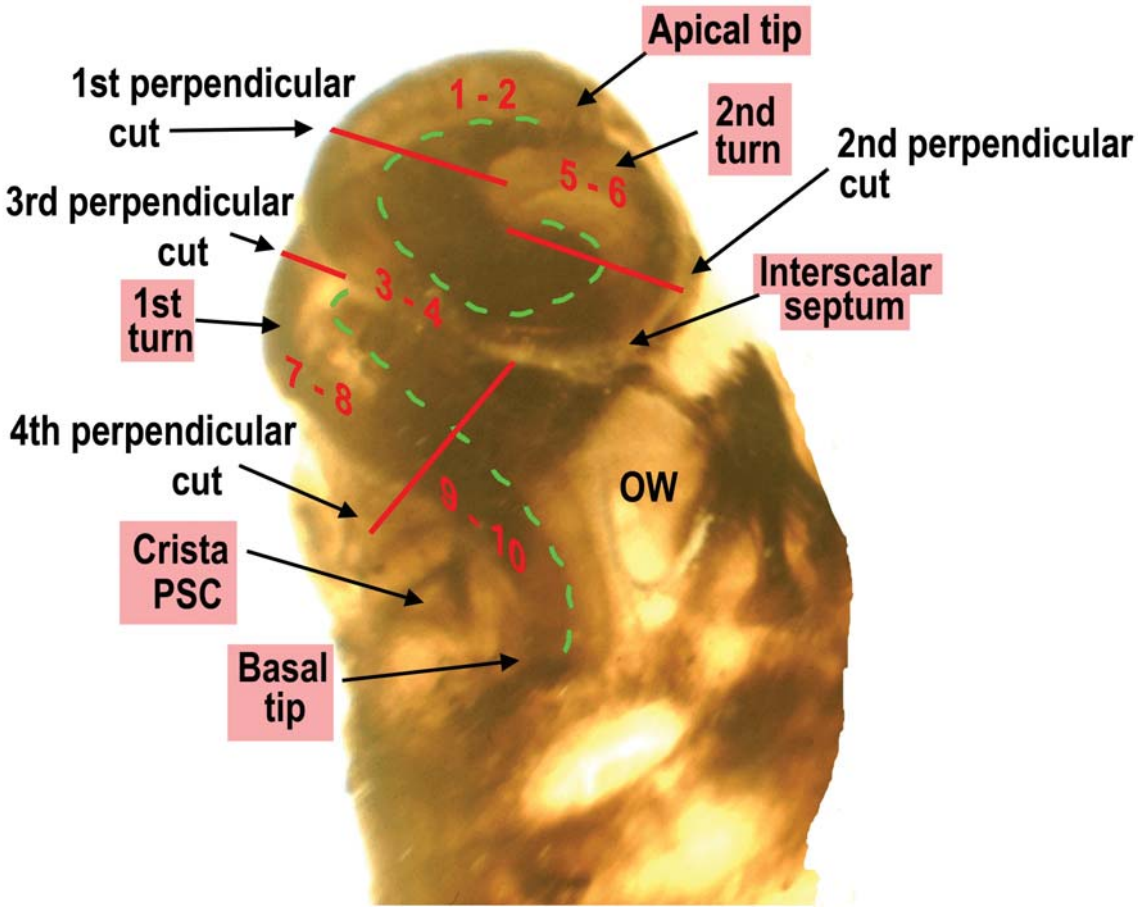


Figure 12: The red lines indicate where to make the perpendicular razor blade cuts that will divide the cochlear duct into five segments that are labeled 1-2, 3-4, 5-6, 7-8 and 9-10. Razor blade cuts that are parallel to the basilar membrane and that pass through the interscalar septum separate each half-turn from the remainder of the specimen. The segments are removed sequentially from apex (i.e., 1-2) to base (i.e., 9-10).

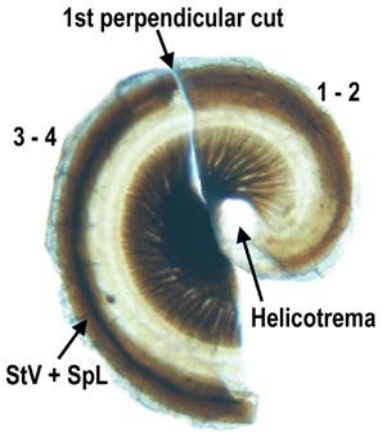


Figure 13: When performing a first dissection of the cochlea, the first perpendicular cut should be made approximately a half-turn away from the apical tip. This cut should be made perpendicular to the basilar membrane from the helicotrema laterally through the organ of Corti, basilar membrane, stria vascularis (StV) and spiral ligament (SpL). The first horizontal cut should be made inferior to segment 1-2, cutting through the interscalar septum and the helicotrema. This cut will separate segment 1-2 from the remainder of the cochlear duct.

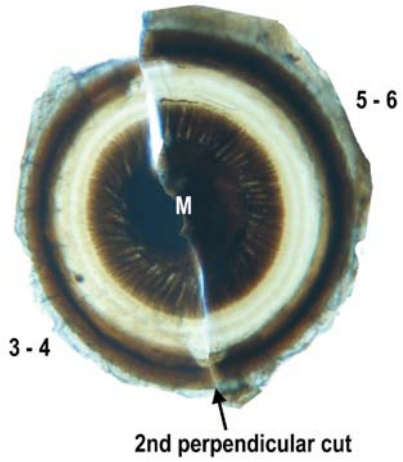


Figure 14: The second perpendicular cut should be made approximately a half-turn away from the first perpendicular cut, cutting through the modiolus (M), the organ of Corti, basilar membrane, stria vascularis and spiral ligament. The second horizontal cut should be made inferior to segment 3-4, cutting through the interscalar septum up to the middle of the modiolus. This cut will separate segment 3-4 from the remainder of the cochlear duct.

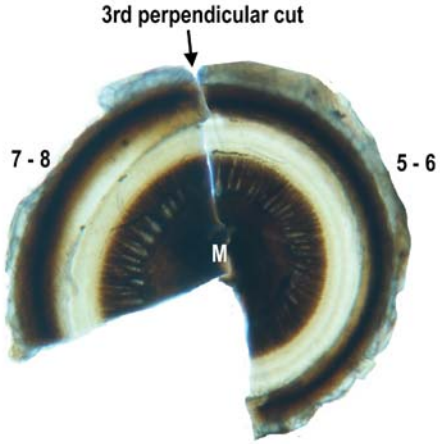
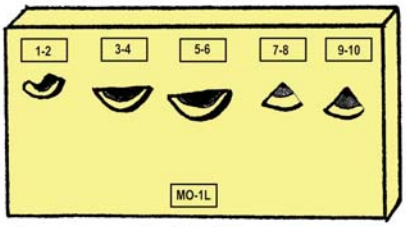


Figure 15: The third perpendicular cut should be made approximately a half-turn away from the second perpendicular cut, cutting through the modiolus (M), the organ of Corti, basilar membrane, stria vascularis and spiral ligament. The third horizontal cut should be made inferior to segment 5-6, cutting through the interscalar septum up to the middle of the modiolus. This cut will separate segment 5-6 from the remainder of the cochlear duct.

Because the cochlear duct changes direction at the base of the cochlea, the relation of segment 7-8 to segment 9-10 cannot be shown here in a 2-dimensional photomicrograph. The fourth perpendicular cut should be placed so as to divide the remainder of the cochlear duct in half to obtain segments 7-8 and 9-10.



Arrangement of half-turn segments & labels in first dissection boat.

Figure 16: Orientation of the half-turns of cochlear duct in the 1st dissection boat. Note that the cut edges of the organ of Corti are near the segment labels. The basilar-membrane side of each segment is facing the bottom of the container.

1ST DISSECTION SEGMENTS OF MOUSE COCHLEAR DUCT

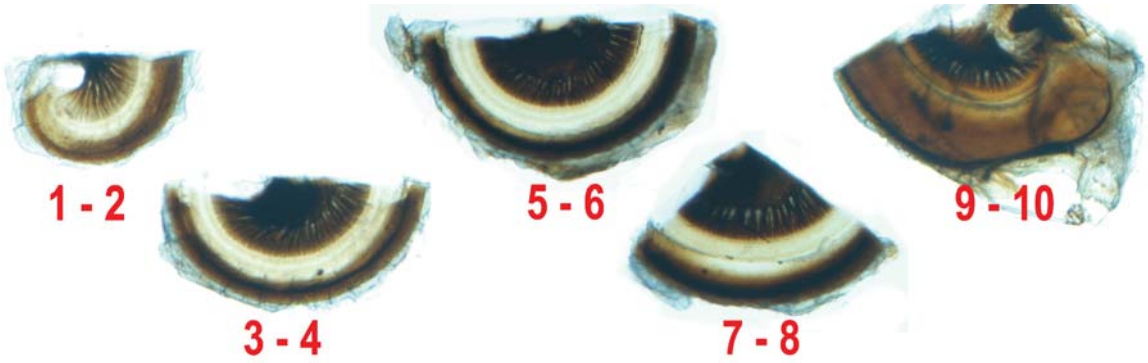


Figure 17: This composite photomicrograph shows five half-turn segments into which the cochlear duct is divided into during the first dissection. The view is through Reissner's membrane. The guiding principle for placement of the perpendicular cuts is to position them so as to not cut through any focal lesions in the organ of Corti. The length of each segment of the cochlear duct will be accurately measured after the second dissection so their lengths need not be a specific length.

In a left cochlea (as shown in Figs. 16 and 17), the upper end of each segment of cochlear duct is to the left when the segments are viewed through Reissner's membrane with the cut edges of the organ of Corti facing away from you. In a right cochlea viewed in the same fashion, the upper edge is to the right. It is important to identify the upper and lower edges of the half-turn segments so that the quarter-turn segments will not be mixed up when the half-turn segments are divided in half.

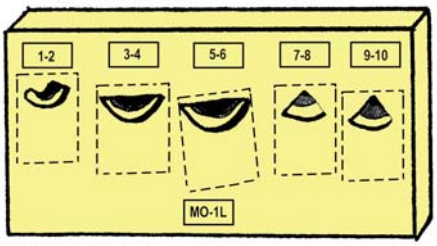


Figure 18: Orientation of saw cuts around OC segments in the 1st dissection block to prepare for the 2nd dissection.

THICK, RADIAL SECTION OF MOUSE COCHLEAR DUCT

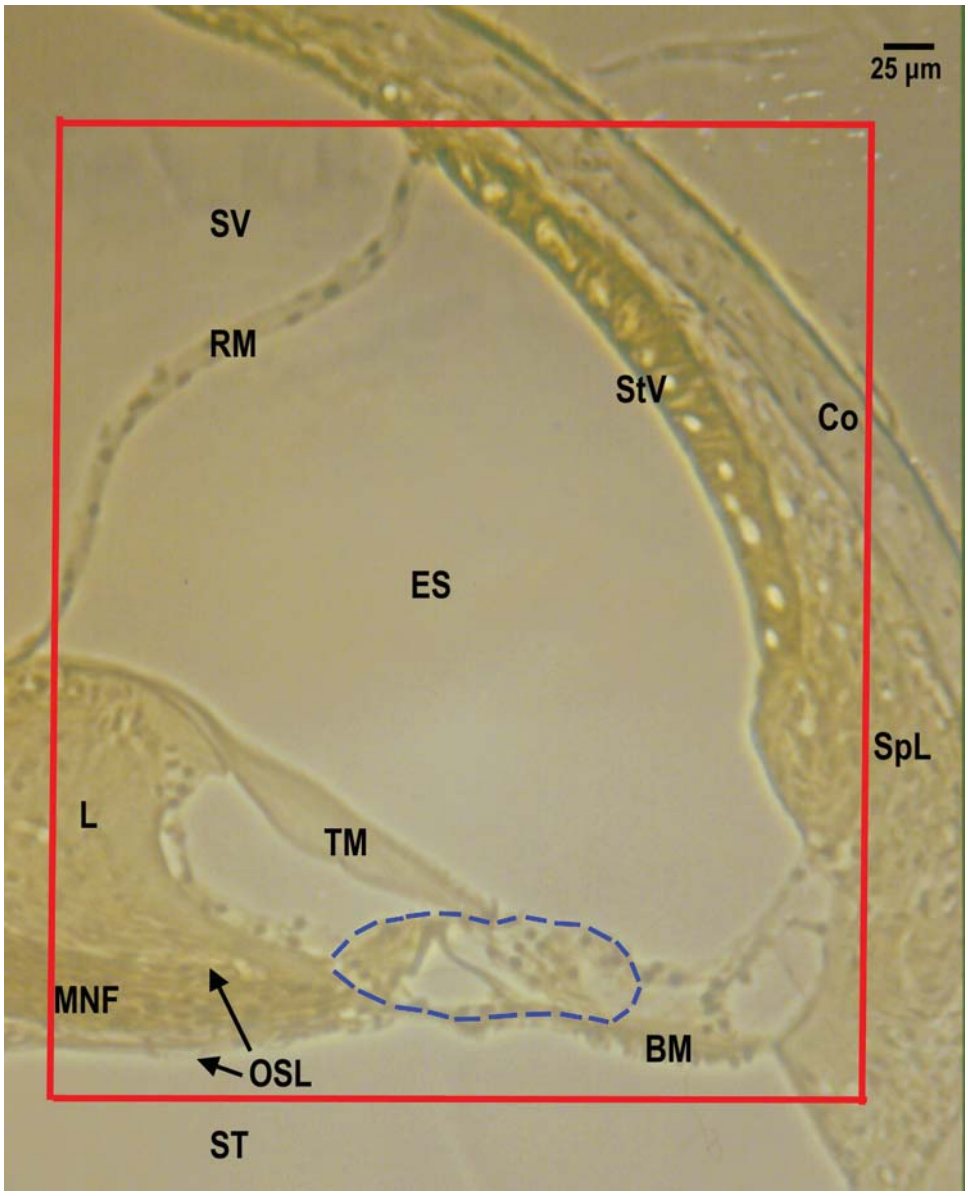
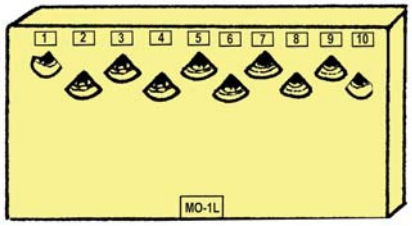


Figure 19: Thick radial section of the cochlear duct upon which the 2nd dissection razor-blade trim lines have been superimposed. Inferiorly, trim close and approximately parallel to the basilar membrane (BM) and the osseous spiral lamina containing myelinated nerve fibers (MNF). Superiorly, remove any remainder of the interscala septum but avoid cutting the attachment of Reissner's membrane (RM) to the spiral ligament (SpL). Medially, leave as much of the limbus (L) and nerve fibers as possible. Laterally, leave the stria vascularis (StV) intact. The blue dashed line delineates the organ of Corti. ES - endolymphatic space; TM - tectorial membrane; ST - scala tympani; SV - scala vestibuli.

2ND DISSECTION SEGMENTS OF MOUSE COCHLEAR DUCT



Arrangement of quarter-turn segments & labels in second dissection boat.

Figure 20: Arrangement of quarter-turn segments of the cochlear duct in the 2nd dissection boat. The basilar-membrane side of each segment is facing the bottom of the boat. Segments are viewed from Reissner's membrane. This is a left cochlea.

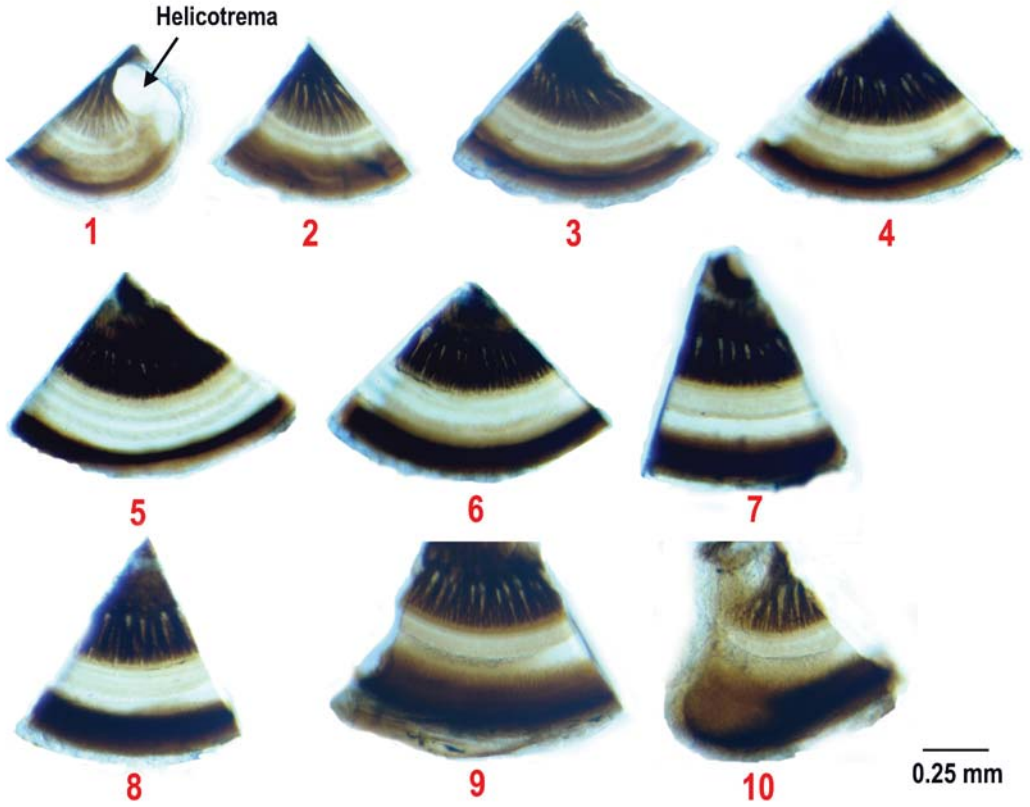


Figure 21: This is a dissection microscope view of the quarter-turn segments of the cochlear duct that were obtained during the 2nd dissection. The view is from the basilar membrane side of the segments. Note that for a left cochlea, the upper end of each segment is to the right when viewed through the basilar membrane with the osseous spiral lamina facing away from you.

2nd DISSECTION of the VESTIBULAR END ORGANS

Remove the 1st dissection boat containing the vestibular sensory organs from the oven and allow it to cool to room temperature before separating the plastic block from the Peel-Away mold.

Unlike the segments of cochlear duct, the vestibular organs need not have a 'handle' when sawed out of the 1st dissection block. For the individual vestibular organs, mark off sawing lines with the diamond-tipped forceps (tips together). Mark off a square as indicated in Figure 22.

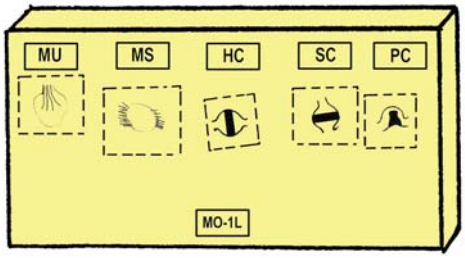


Figure 22: Appearance of vestibular end organs in 1st dissection boat, along with lines for sawing organs out of block for subsequent dissection. (The organs are not drawn to scale.)

Place the saccular (or utricular) macula on the glass plate of the dissection microscope and coat with liquid araldite. Use quarter pieces of razor blades to trim the plastic around the macula as well as over the endolymphatic surface and on the opposite side where the bone of the vestibule is located. The trimming should be close to the macula and parallel to the sensory epithelium and otoconial layer. Trim away the non-sensory epithelium of the macula but do not trim away any of the otolithic layer, sensory epithelium or the nerve fibers that are entering the epithelium. When finished, the intact saccular (or utricular) macula should be in a small plastic block and the otoconial layer or endolymphatic surface of the epithelium should be clearly visible. This block can either be re-embedded whole in a 1.5-mm-thick layer of plastic (Fig. 23) or can be split into 3-4 hand-cut, thick razor sections before re-embedding (Fig. 24). If the organ is left intact, embed it so that the otoconial layer faces the bottom of the container.

To split the maculae, hold them in a pool of araldite on the glass plate with the otoconial layer facing up. Use a new quarter blade to separate off 1/4 of the macula. Try to cut perpendicular to the sensory epithelium. Put the thick section in a R-40 Peel-Away mold containing 1.5 ml of liquid araldite. Orient the section with the cut surface facing the bottom of the container. Split the remainder of the macula into 2-3 more thick sections. Place each section in the Peel-Away mold. When finished with one macula, prepare a boat for the other macula.

The cristae should be handled in a similar fashion with the sensory epithelium split perpendicular to the long axis of the epithelium (Fig. 25) using quarter razor blades. Polymerize the boats for 48 h at 60° C.

WHOLE MOUNT VIEWS OF MACULAE OF SACCULE & UTRICLE

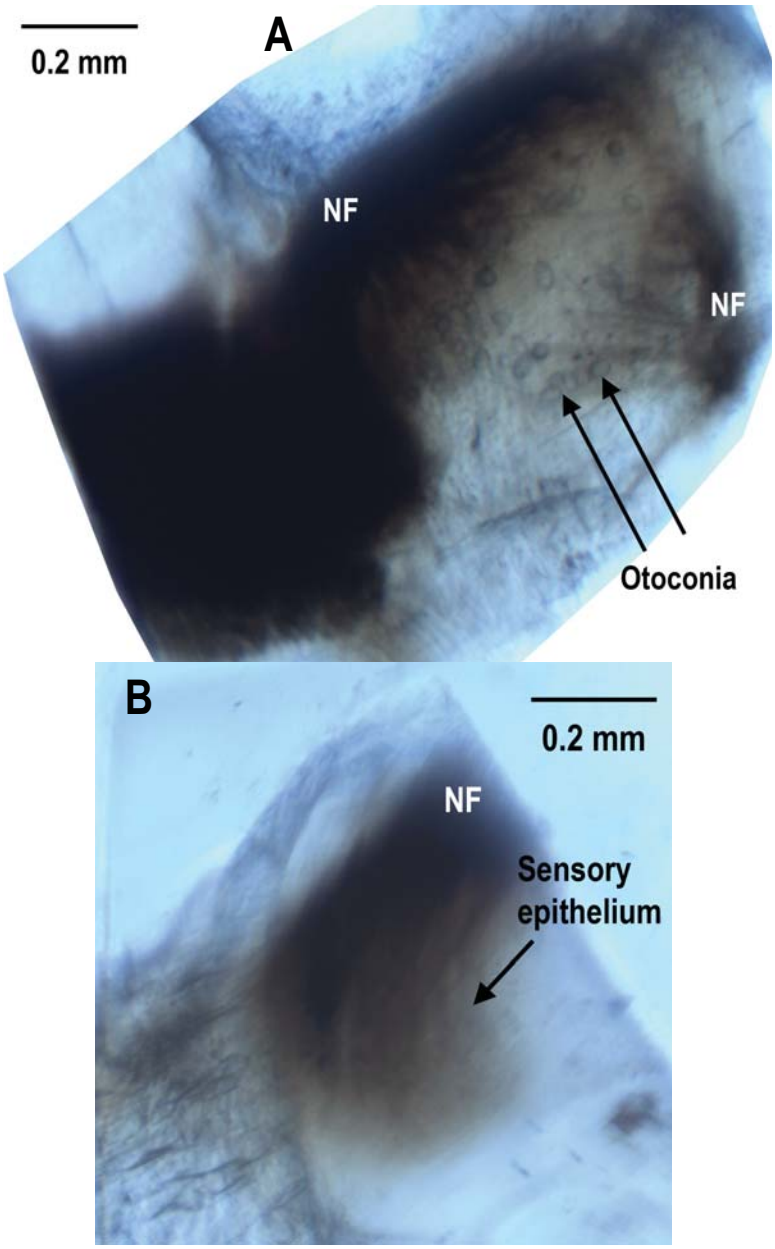


Figure 23: Dissection microscope appearance of the saccular macula (A) and utricular macula (B) viewed from the endolymphatic surface. Nerve fibers (NF) can be seen entering the sensory epithelium. These organs are from a mouse with a vestibular mutation. Instead of a normal complement of small otoconia, there are about 20 moderate-sized otoconia over the saccular macula. Note these specimens were not decalcified.

THICK, RADIAL SECTIONS of MACULAE of SACCULE & UTRICLE

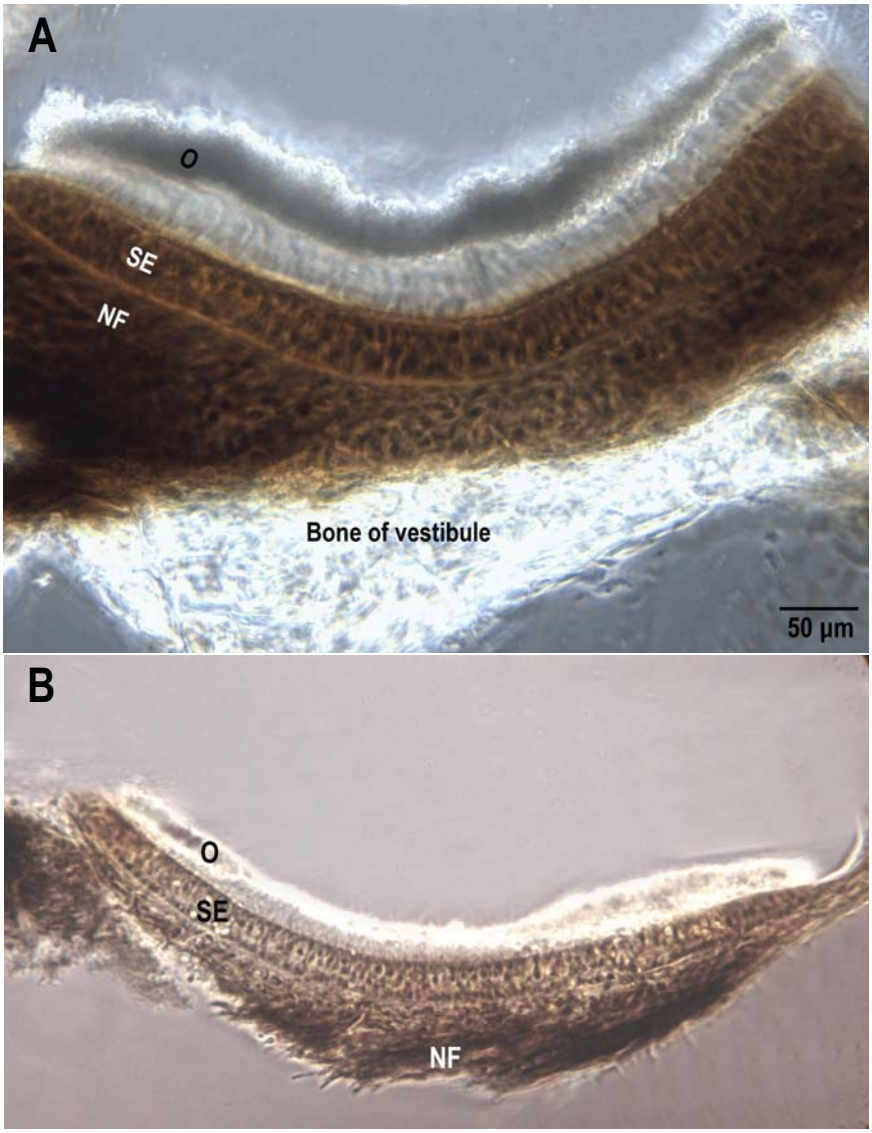


Figure 24: Hand-cut, thick radial sections through the maculae of the saccule (A) and utricle (B) from a normal mouse. For the saccular macula, the sensory epithelium (SE) lies close to the bone of the vestibule. For the utricular macula, the sensory epithelium (SE) is located near the center of the vestibule. Myelinated nerve fibers (NF) enter the epithelium from its inferior surface. A layer of otoconia covers the endolymphatic surface of the epithelium. Note these specimens were not decalcified.

THICK, RADIAL SECTION of CRISTA

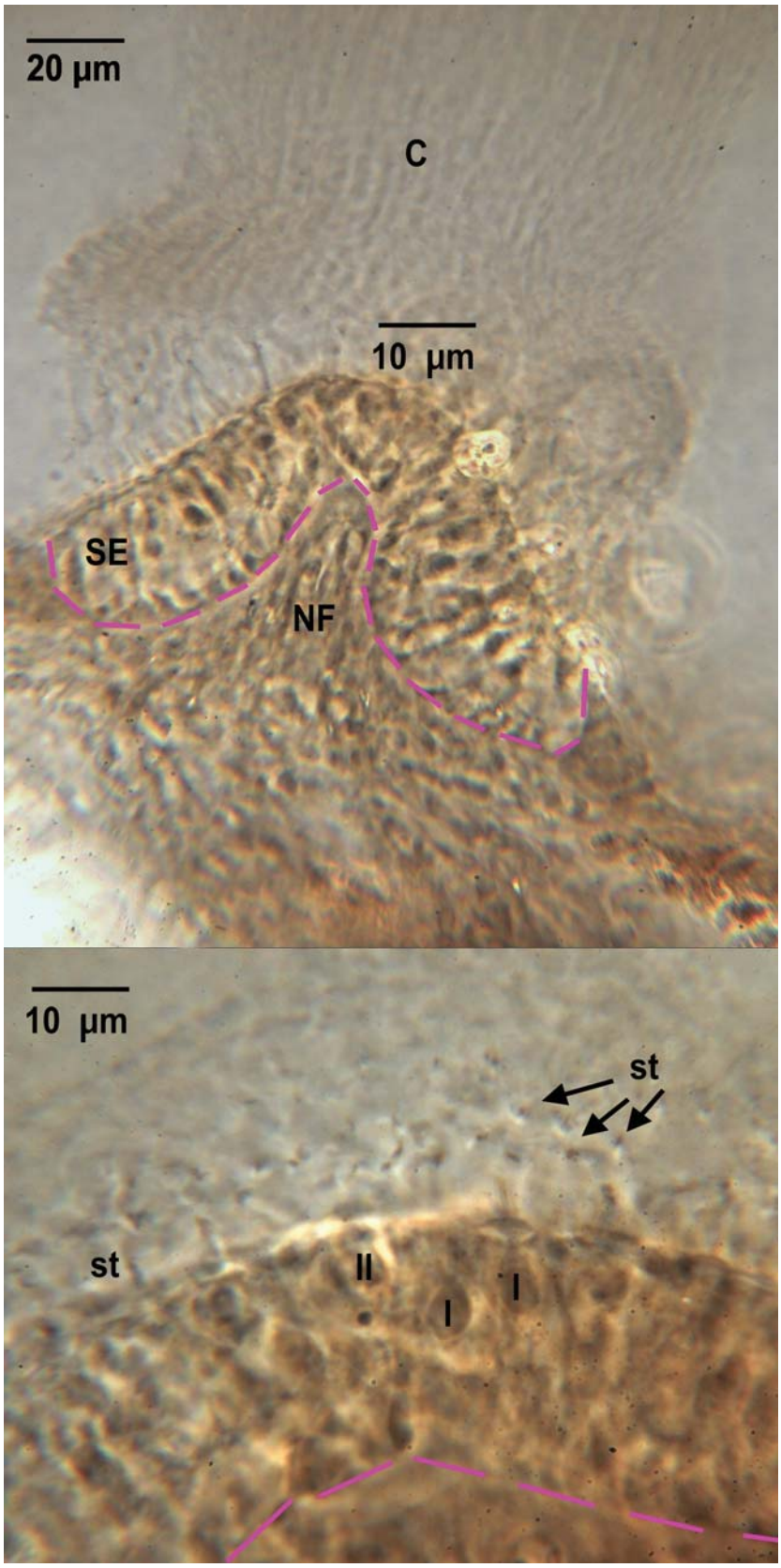


Figure 25: Thick razor section of a crista that was cut perpendicular to its long axis. The boundary of the sensory epithelium (SE) is indicated by the pink dashed line. It covers a mound of connective tissue through which the nerve fibers (NF) enter to innervate the hair cells. The stereocilia (st) on the type I (I) and type II (II) hair cells extend into the cupula (C), an extracellular gelatinous material.

After the blocks containing the thick sections of maculae and cristae are polymerized, the sections can be examined at a high magnification by phase contrast microscopy (Fig. 26). The blocks can also be sectioned with an ultramicrotome and stained for light-microscopic examination (Fig. 27).

PHASE-CONTRAST VIEW of RADIAL SECTION of SACCULAR MACULA

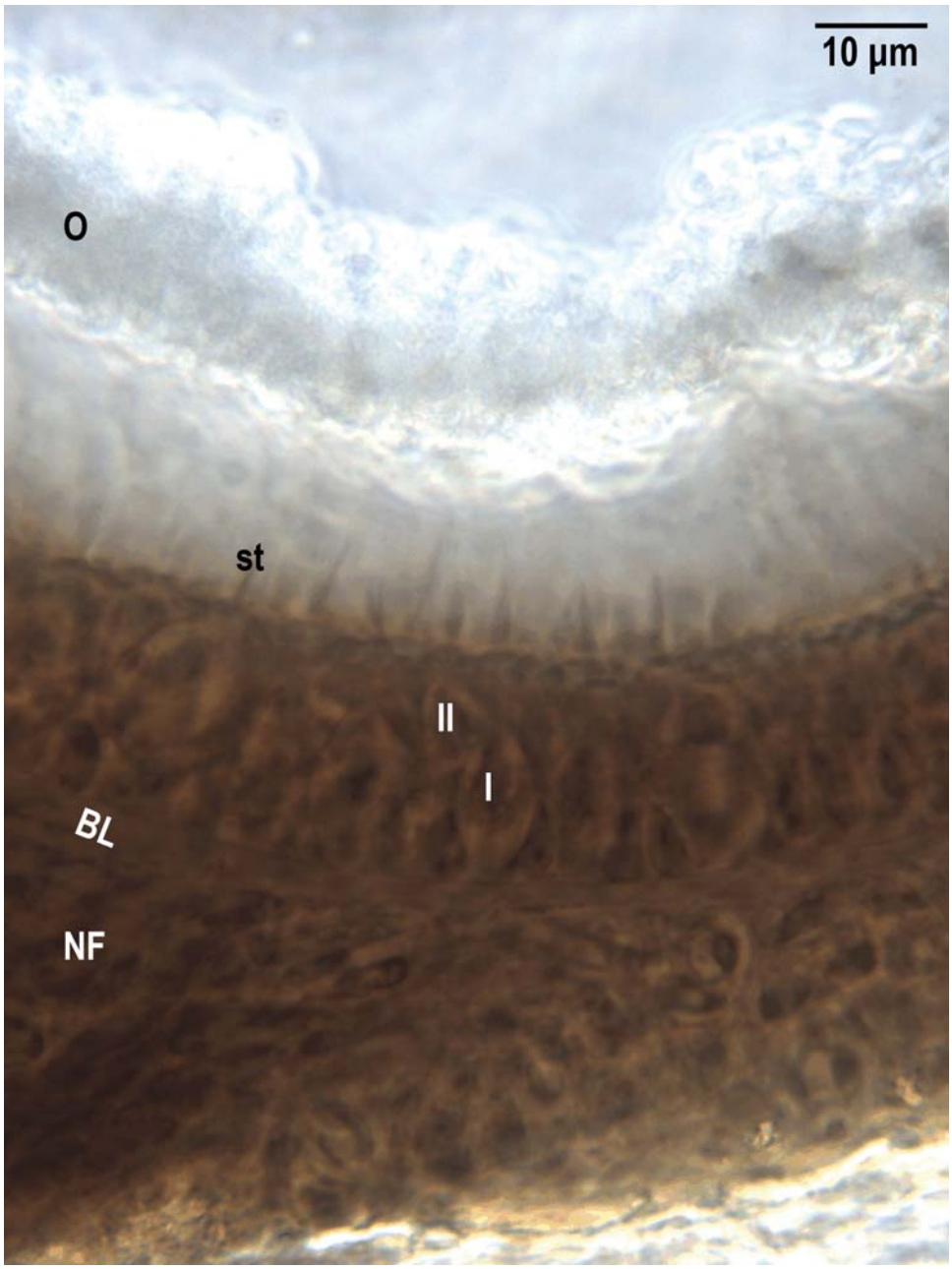


Figure 26: Higher magnification of thick radial section of the saccular macula. The sensory epithelium contains type I (I) and type II (II) hair cells. Bundles of stereocilia (st) can be seen projecting from the apical surface of each hair cell. A mass of otoconia overlies the sensory epithelium. The epithelium is separated by the basal lamina (BL) from the underlying connective tissue that contains nerve fibers (NF).

1-MICROMETER-THICK, RADIAL SECTION of SACCULAR MACULA

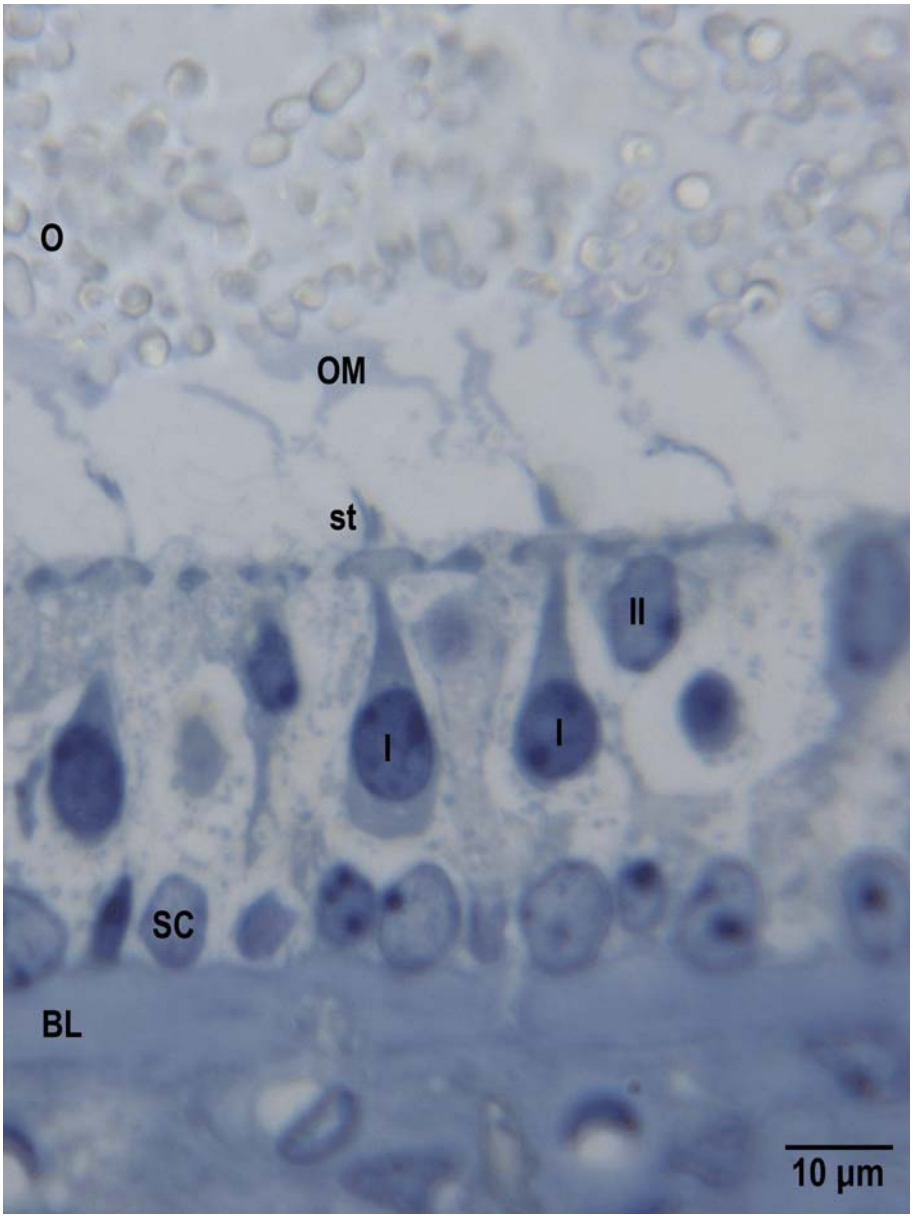


Figure 27: Stained,1-µm-thick section of the saccular macula from a normal mouse. The sensory epithelium consists of hair cells (type I and type II) and supporting cells (SC). Type I hair cells are flask-shaped and their basolateral surfaces are entirely surrounded by an afferent nerve terminal called a nerve chalice. Type II hair cells have shapes similar to short outer hair cells. Their nuclei are near the epithelial surface. Supporting cell nuclei (SC) form a nearly continuous line adjacent to the basal lamina (BL). Otoconia (O) are embedded in the otolith membrane (OM), an extracellular gelatinous material covering the sensory epithelium. This specimen was not decalcified. (Methylene blue - Azure II stain).

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ACKNOWLEDGMENTS

Dr. David M. Ornitz provided many of the mice that were used to develop the preparation techniques that are described in this manual. We gratefully acknowledge the excellent technical assistance provided by Ms. Patricia A. Keller who processed and sectioned the mouse temporal bone illustrated in Figure 6.

APPENDIX A: Preparation of 1% Osmium Tetroxide in Dalton's Buffer with Calcium Chloride

1. Make up the following solutions using demineralized, distilled water in separate flasks:

   a) 2.5 N KOH (7 g/50 ml H_2O)

   b) 5% $K_2Cr_2O_7$ aqueous solution (2.5 g/50 m1 H_2O)

   c) 3.4% NaCl aqueous solution (1.7 g/50 m1 H_2O)

2. Adjust the pH of the $K_2Cr_2O_7$ to pH 7.6 by adding 2.5 N KOH (usually between 7.2 and 7.5m1). Keep track of # of ml added.

3. Dilute the adjusted $K_2Cr_2O_7$ (see # 2) from 5 % to 4 % by adding the appropriate amount of demineralized distilled water. For example:

$x = \frac{50 \times 5}{4}$, where x=total # ml in 4% solution

Therefore, x = 62.5 ml

50 m1 5% $K_2Cr_2O_7$ + # ml 2.5 N KOH + y ml H_2O = 62.5 ml 4% K_2CrO_7

4. Put 25 ml of $K_2Cr_2O_7$ (4 %) into each of two flasks. Add 2 5 ml of 3.4% NaCl aqueous solution and mix.

5. Add 50 ml of H_2O to each of the flasks in (4). Mix well. Check the pH of each. It should be close to 7.3.

6. To each flask, add 1cc of 1.65% CaC12 drop by drop shaking after each drop.

7. Put solution from each flask into two glass stoppered bottles. While working in a fume hood, snap the top off of a glass vial (the vial is prescored) containing 1 g of osmium tetroxide (OsO_4) and put one into each bottle. Be careful not to inhale the vapor or spill the solution; OsO_4 is poisonous. This solution should be kept in the fume hood and should be shaken frequently at room temperature until the OsO_4 is dislodged from the vial. Sonicate the solution for 15 minutes to speed up the osmium dissolving in the buffer.

8. When the OsO_4 crystals are dissolved, cover the top of the bottle with Parafilm and tape securely to the bottle neck. Store the bottles in the freezer in room 1145. This prevents oxidation of the OsO_4.

9. No matter what quantity of liquid OsO_4 is needed, the entire container of fixative must be completely thawed for the concentrations to be correct. To thaw the solution quickly, the bottle can be placed in a container of warm water. Be careful not to continue warming too long; the OsO_4 will oxidize. Be certain to swirl the bottle after thawing to make the osmium concentration uniform in the bottle. After removing the needed amount of fixative from the bottle, re-cover, tape and re-freeze the bottle for future use.

APPENDIX B: Sharpening the Pick

Picks are made from a pin vise (54270 - Ernest F. Fullam, Inc; or 26015-11 - Fine Science Tools) and a straight sharpened needle.

It is essential to have well-sharpened picks. The pick works best if its point is formed by three equal sized triangles. The beveled edge between two sides is generally as useful as the point. An Arkansas stone with one or two drops of oil on it is used for sharpening. Sharpening should be done under the dissection microscope (40X).

The pick is sharpened by a forward and backward motion (not lateral) on the stone. The angle at which the pick is held will determine the area of the face being made. A steep angle results in small faces and a broad tip while a shallow angle results in long faces and a narrow tip. It is best to finish one side before sharpening the next. If one edge of the last side made is kept straight up, it is easier to form three sides of approximately equal area. When all three sides are completed, the edges and point should be sharp and fine with the tip centered over the shaft of the pick. If they are not, further sharpening should be done.

Be certain to wipe off the oil on the pick with an alcohol prep pad before using it.

APPENDIX C: Preparing glass pipettes

Fifteen cm lengths of 3 mm 0.D. (wall 0.75 mm) glass tubing are cut by scoring the tubing at the appropriate place and snapping it. After approximately 30-40 lengths have been made, wash them in acetone, followed by soapy water. Dry the tubes in the 37° C oven. The tubing must be thoroughly dry before making pipettes.

Grip the ends of one glass tube with the thumb and forefinger of each hand and rotate it over the flame of a Bunsen burner. When the center of the glass tube is soft, evenly and quickly pull the ends apart by about two feet. The two sides are then separated at the center where the tubing is very thin. Be careful not to touch the heated portion of the tube; it is very hot. If the tubing is pulled too quickly, if it is not removed from the flame before pulling, or if it is pulled too long after the tube has been removed from the flame, the thin portion will not be straight, or it will not be thin enough. These tubes cannot be used.

When a number of these "pipettes" have been made, tips must be put on them by pulling them a second time. The thin portion of the pipette which is to be pulled should be within 1-2 mm of the thick portion of the glass tube. It is best to move the pipette in and out of the flame of an alcohol burner repeatedly. The thin portion of the glass tube takes much less time to heat up and become soft. If the thin tube is left in the flame too long, its lumen will collapse. The pulling should be done when the glass is soft and out of the flame. It is important that the two ends remain joined after pulling until the glass cools so that the pipette remains straight. The pipette tip is made by carefully snapping the thinned tube off at the desired point with the nails on the thumb and forefinger.

It is important to look at the pipette tips under the dissection microscope at 40X to determine whether they have flat ends or slanted ends. The slanted ends will not allow the proper sealing of the perfusion hole for adequate fixation of the cochlea. The tip diameter must approximate the size of the perfusion hole. At least ten pipettes should be available for use before anesthetizing the mice for fixation.

There will probably be some pipettes with tip diameters that are too large for perfusion. These can be used for removal of bone chips and bubbles when the cochleae are in 70% alcohol or araldite.

The size and length of the tapered portion of the pipette are important. If the tapered portion is too long and thin, it will take a long time to pull the OsO_4 into the pipette. It also may be too flexible; this will make it more difficult to hold the pipette perpendicular to the perfusion hole. It should not be curved for the same reason.

Once you are satisfied with the pipettes, wrap the non-tapered end of each pipette with a 1.5 cm length of waterproof tape. This will provide a non-slip seal between the pipette and the latex perfusion tube.

APPENDIX D - Durcupan (Araldite) preparation

Araldite should be made at least 2 hours before it is needed so that the bubbles can be removed before the plastic is needed. If the solution is used before the bubbles dissipate, they may get into the tissue and result in poor embedding. To avoid wasting araldite, carefully calculate the amount needed.

Do not make more than 50 ml of araldite per batch. Larger quantities are harder to mix thoroughly and tend to get quite dark.

Ingredients	Amount
A - epoxy resin	10 cc
B - hardener	10 cc
C - accelerator	0.35 cc
D - plasticizer	0.40 cc

1. To measure components **A** and **B**, use the graduated cylinders which are designated for araldite preparation only. They are kept in a wire basket to the right of the hood (rm 2110).

2. Pour components **A** and **B** into the same graduated cylinder, **B** following **A** (try to pour in center of the cylinder so that the plastic does not adhere to the side).

3. Pour components **A** + **B** into an appropriate sized disposable plastic beaker after blowing the dust out with a microduster. Allow graduated cylinder to drain for five minutes into plastic beaker to get most of araldite out of the cylinder.

4. Use the 1000 µl pipetter to measure first **D**, then **C**. Add C last because mixture will begin to polymerize with its addition. After adding C, promptly begin to mix components (see step 5).

5. Stir the plastic mixture vigorously with the hand mixer until the mixture is frothy with air bubbles and is thoroughly mixed (at least 2 minutes). Cover with Parafilm until needed. One can speed the disappearance of the bubbles in the freshly made araldite by putting the mixture in a 50-ml plastic centrifuge tube and centrifuging at a low speed for 5 minutes. Araldite can be kept in the refrigerator and used for re-embedding in the molds. If the previously prepared araldite does not flow freely after sitting at room temperature for about an hour, it has begun to polymerize and should not be used.

6. Use a 25 ml graduated cylinder to mix a total of 12 ml for each of the dilutions needed for the cochleae from three mice (i.e., 3 vials). Mix the dilutions about ½ hour before needed:

8 ml propylene oxide: 4 ml araldite = 2:1 dilution
6 ml propylene oxide: 6 ml araldite = 1:1 dilution
4 ml propylene oxide: 8 ml araldite = 1:2 dilution

Pour propylene oxide into the cylinder first, followed by the plastic. Mix with a fire-polished glass rod until the araldite is completely dissolved (no threadlike strands of araldite are visible). The same graduated cylinder can be used for all three dilutions without washing.

7. After use, pour the araldite dilutions and used araldite into a disposable container. Do not pour them into the sink.

8. Invert the graduated cylinders on a paper towel to allow the excess araldite to drain out. Wash with acetone to dissolve araldite. Soak araldite-impregnated cork stoppers in acetone, wipe off glass stirring rods with acetone on Kimwipes. Wash graduated cylinders and rods in hot water glass washing soap. Rinse first with tap water and then with distilled water. Allow to drip dry.

9. Amounts of araldite needed for processing 6 temporal bones from three mice:

a) 18 ml - propylene oxide/araldite mixtures (see point #6)
b) 48 ml - 4 ml for each fresh araldite change (4 ml for four changes in three vials).
c) 6 ml - 2 ml for each medium-sized Peel-Away mold (3 molds needed for three mice).

Total - 18 + 48 + 6 = 72 ml. Round to 80 ml.

APPENDIX E: Razor blade preparation

1. To break a whole blade (Fig. 1E) into quarters, pick up blade by its non-cutting edges and break it in half lengthwise (Fig. 2E - dashed line). When snapping blades in half, try to keep sharp edges from hitting one another and getting damaged. Take one of the halves and break in half again by folding the two non-cutting edges toward one another until the blade breaks. This will result in two quarter blades; one "forwards" quarter and one "backwards" quarter. Figure 3E illustrates forwards and backwards quarter blades.

2. To obtain middle blades, break the ends off (Fig 4E) then break each “middle” into 2 equal lengths. The resulting rectangular shaped pieces with no handle are the "middle" blades. The remaining pieces should be discarded in a sharps container.

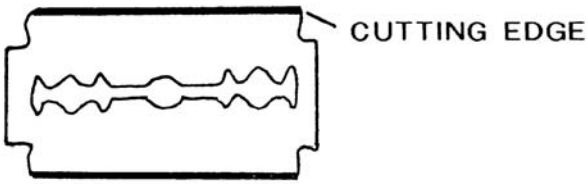

Figure 1E: Unbroken, double-edged razor blade.

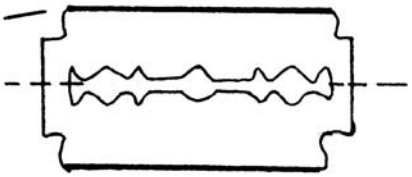

Figure 2E: Dashed line indicates first break to make when preparing quarter razor blades.

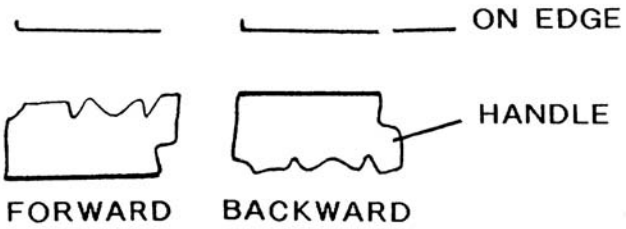

Figure 3E: Quarter blades

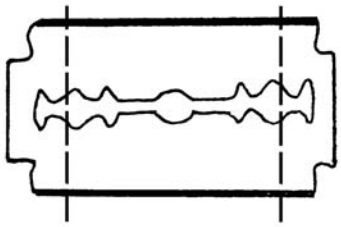

Figure 4E: First breaks to prepare "middle" blades

All broken edges of middles should curve the same way. This is accomplished by making all folds in the same direction.

3. Place quarter or middle blades in appropriately labeled dust-free plastic or metal boxes to prevent damage to their cutting edges.

APPENDIX F: Aligning the phase-contrast microscope for examination of plastic-embedded flat preparations

1. Use sharp quarter blades or a large, sharp scissors to trim edges of specimen blocks so that blocks will lie flat when placed dull side up (i.e., labels upside down). If block has been polymerized for 48 hours, a bench grinder (room 2112) can be used to flatten the edges.

2. Put the trimmed block into aluminum holder and secure in place with cellophane tape. Tape at edges away from the segments of cochlear duct. Place aluminum holder in slide holder on microscope stage. Orient block so that modiolar edge of segments is towards you.

3. Put a droplet of immersion oil over the segment that is to be examined microscopically.

4. Rotate phase plate of microscope to 10 and objective to 10X..

5. Roughly focus on a segment with the coarse focus adjustment of the microscope.

6. Close down the field diaphragm until it is visible in the microscope field.

7. Move the condenser up or down until image of field diaphragm is in focus in the plane of the specimen. If the condenser is too low, the center of the field will appear dark red; if the condenser is too high, the center of the field will appear blue. When adjusting the position of the condenser, always try to make the center of the field colorless. If the specimen block is too thick, it will be impossible to get the condenser focused on the specimen. In this case, raise the condenser as high as it will go.

8. Center the light by moving the lamp housing below the specimen stage while observing through the binocular eyepieces.

9. Open the field diaphragm just enough to fill the field with light.

10. Remove the right eyepiece and replace with a telescoping eyepiece.

11. Adjust the telescope until the phase rings are in sharp focus for your right eye.

12. Use left and right spring-loaded pins to move phase plate so that the annulus in plate overlays the annulus in the objective.

13. Replace the telescoping eyepiece with the regular right eyepiece. The segment is now ready for viewing at 100X by phase contrast microscopy.

14. If you need to examine the specimen with a higher power objective, steps 4 through 13 must be followed again for the appropriate numbered phase plate and objective lens.

15. The 50X and 100X objective lenses are oil immersion lenses and should be immersed in the oil droplet over the segment. The 10X, 20X and 40X objective lenses are not oil immersion lenses. The view will appear fogged if you get oil on these lenses. If you accidentally get oil on these lenses, use soft lens paper to clean them off immediately.

APPENDIX G: Cochlear perfusion apparatus

Many fixatives, including 1% osmium tetroxide in potassium dichromate buffer, are toxic. Avoid breathing fumes from the bottle of fixative when filling the specimen vials by using a fume hood. It is nearly impossible perform in-vivo fixation of the cochleae inside a fume hood because of the need for an operating microscope. Therefore, when filling and emptying the perfusion pipette, use the perfusion apparatus illustrated below (Fig. 1G). Keep the stoppered specimen vials containing fixative in an ice bath when not filling the perfusion pipette.

The perfusion apparatus consists of a short, heavy-walled glass jar (approximate capacity 30 ml) containing about 5 ml of corn oil into which is fitted a #7 rubber stopper. Two 3-mm diameter holes are drilled through the stopper and a short length of 3-mm glass tubing is fitted into each one. The pieces of glass tubing project about 1 centimeter above the top of the stopper but end at the bottom of the stopper. Two lengths of latex tubing (1/8" ID) are fitted to the glass tubing. The tubing must be wrapped with 1.5-cm lengths of waterproof tape for a snug fit between the latex and glass. One length of tubing is fitted with a mouthpiece that is used to increase or decrease pressure in the glass jar. The other length of tubing is fitted to a perfusion pipette. By decreasing pressure in the jar, fixative can be pulled into the pipette. By increasing pressure in the jar, fluid in the pipette can be expelled in a controlled fashion. Any fixative leaving the tubing connected to the perfusion pipette will be trapped in the glass jar and oxidized by the corn oil. Thus, you will not risk ingestion of fixative during cochlear perfusion.

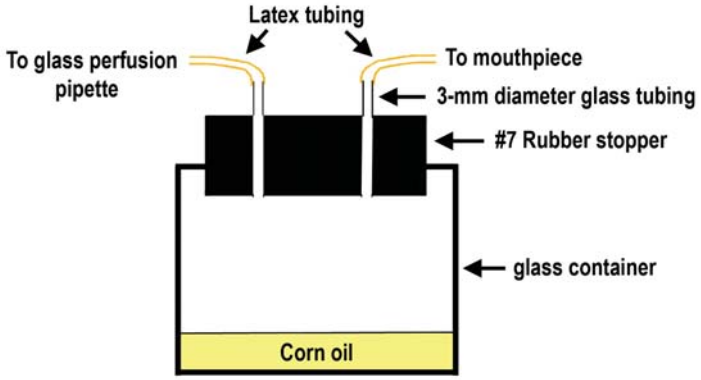

Figure 1G: Perfusion apparatus to minimize the hazard of working with toxic fixatives during in-vivo fixation of the cochlea. The corn oil in the glass jar absorbs and oxidizes osmium tetroxide.