CellPress

Protocol

High-resolution imaging of the mouse-hair-cell hair bundle by scanning electron microscopy

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Highlights

Histology and dissection of the mouse's inner ear sensory epithelium

Sample preparation for scanning electron microscopy of the hair cells

Allows imaging of the mechanotransduction organelle and the tip links

Scanning electron microscopy (SEM) allows cell surface imaging at a sub-nanometric resolution. However, the sample requires a specific preparation to sustain the high vacuum of the SEM and be electrically conductive. The sample preparation consists of dissection, fixation, dehydration, metal coating, and tissue mounting. Here we provide a comprehensive protocol to perform SEM on the mouse's inner ear, and image the hair bundles at high resolution. Hair bundles are the force-sensitive organelles located at the apical surface of hair cells.

Protocol

High-resolution imaging of the mouse-hair-cell hair bundle by scanning electron microscopy

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SUMMARY

Scanning electron microscopy (SEM) allows cell surface imaging at a sub-nanometric resolution. However, the sample requires a specific preparation to sustain the high vacuum of the SEM and be electrically conductive. The sample preparation consists of dissection, fixation, dehydration, metal coating, and tissue mounting. Here we provide a comprehensive protocol to perform SEM on the mouse's inner ear, and image the hair bundles at high resolution. Hair bundles are the force-sensitive organelles located at the apical surface of hair cells.

For complete details on the use and execution of this protocol, please refer to Trouillet et al. (2021).

BEFORE YOU BEGIN

Anatomical features central to tissue preparation

The Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University (APLAC protocol #28278) approved all animal procedures involved in this study. Before using this protocol, researchers must acquire authorization to perform animal work from their relevant institutions.

This procedure has been developed for use on tissues with externalized cell features or surfaces of interest; our special focus is on the hair cell bundle–which consists of apical, actin-filled cellular protrusions called stereocilia–but the protocol can also be applied to any tissues with other types of cellular protrusions, such as primary cilia or microvilli.

The inner ear is a complex organ, and strong anatomical knowledge is necessary for its successful dissection (see Figure 1): Sound waves enter the outer ear and vibrate the eardrum at the entrance of the middle ear (Figure 1A). Attached to the eardrum is a chain of three ossicles that transmits the eardrum vibrations to the inner ear. The last ossicle, the stapes, acts as a piston on the oval window, a small membrane-sealed opening in the cochlear bone (Figure 1B). A difference of pressure occurs between fluid-filled compartments in the cochlea (scala vestibuli and scala tympani), generating a mechanical stimulation to the middle chamber (scala media) containing the sensory epithelium (Figures 1C and 1D). High-frequency sounds are detected at the base of the cochlea and the low-frequency sounds at its apex. The mechanosensitive cells of the cochlea, the inner and outer hair cells, are covered by a gelatinous membrane, the tectorial membrane (Figure 1E). The organelle of mechanotransduction of hair cells is the hair bundle. The hair bundle consists of an assembly of membrane protrusions filled with F-actin, the stereocilia, organized in rows of increasing heights (Figures 1F and 1G). An external filament, the tip link, connects the tip of a stereocilium to the side of its taller neighbor (Figure 1H). Deflection of the hair bundle will increase tension in the tip links, opening mechanosensitive ions channels and ultimately depolarizing the hair cell.
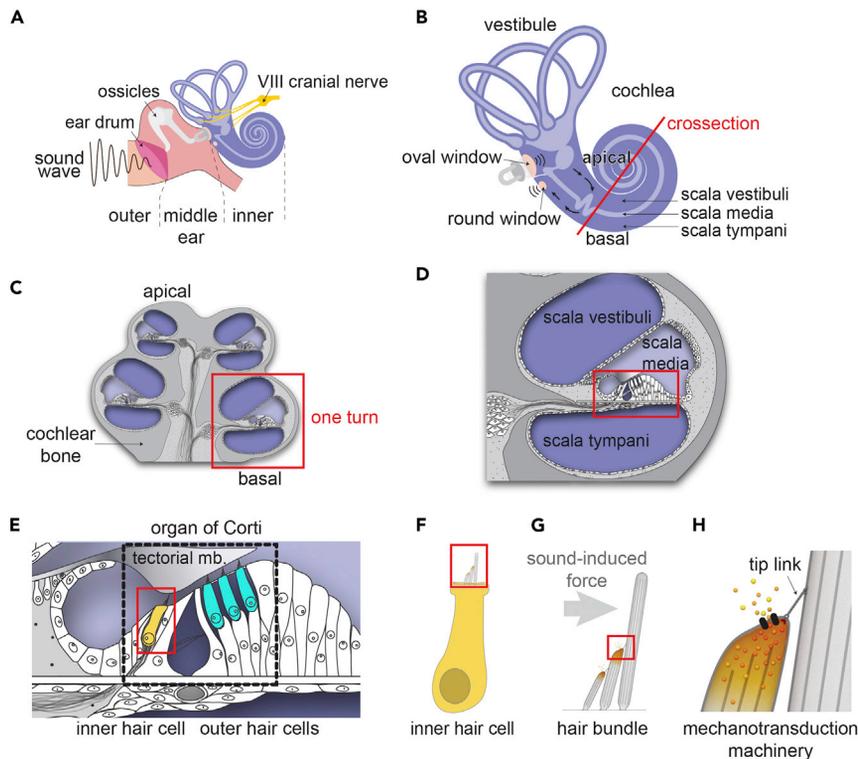
Figure 1. Anatomy of the mammalian auditory organ and its sound-sensitive cells

(A) Sound waves vibrate the eardrum. The vibrations are transmitted to three ossicles which will push back-and-forth onto the inner ear at the level of the oval window, a membrane-covered opening. Graphics of the inner ear were adapted with permission from Shutterstock.

(B) The pressure exerted at the oval window creates a traveling wave inside the fluid-filled cochlea, the part of the inner ear dedicated to sound detection. The traveling wave will progress along the cochlea and mechanically stimulate the central duct (the scala media) and dissipate at a given location depending on sound frequency: at the cochlea base for the high-frequency sounds and the cochlear apex for the low-frequency sounds.

(C) A central cross section of the spiral-shaped cochlea reveals the different fluid-filled ducts (or scala) running along the cochlea length.

(D) The auditory epithelium, or organ of Corti, is located at the inferior part of the scala media.

(E) The organ of Corti contains mechanosensitive hair cells. The inner hair cells (colored in yellow) are the main auditory cells, releasing neurotransmitters to afferent neurons. The outer hair cells (colored in cyan) are present in three consecutive rows and are dedicated to sound amplification and frequency selectivity. At their apical surface, both hair cell types have a membrane specialization called the hair bundle, which is the organelle of mechanotransduction. In addition, an acellular membrane, the tectorial membrane, lies on top of the hair bundles.

(F) the hair bundle consists of actin-filled membrane protrusions organized in rows of increasing heights.

(G) Stereocilia of different rows are connected from the tips of the shorter ones to the sides of a taller one by an extracellular link.

(H) These extracellular links, called the tip links, transmit the deflection force of a tall stereocilium to the tip of a shorter one, where mechanosensitive ion channels reside. Potassium and calcium enter the stereocilia and depolarize the hair cells, leading to synaptic release. Disruption of the tip links or the hair bundle prevents mechanotransduction and leads to hearing loss.

The inner ear dissection is easy between postnatal day (P) 0 and P7. However, after P7, the external surface of the inner ear ossifies, which makes the dissection more challenging. Therefore, we recommend learning the procedure with tissue from P0-P7.

Experimental design

If the experimental goal of your SEM is to characterize a phenotype, always include a negative control animal (wild-type or untreated) from the same litter in the experiment. We recommend at least three animals per condition or genotype at a given age. Ideally, the experiment should be

performed blindly, without knowing the sample's group or genotype. The experiment should be replicated at least two additional times if a phenotype is found.

Toxicity of reagents

The fixatives used in this protocol require wearing appropriate protective eyeglasses, long-sleeved clothing, and disposable gloves. Paraformaldehyde aqueous solution can cause skin irritation. Glutaraldehyde is even more toxic, and you should avoid inhalation. Open the glass vials containing the fixatives under a fume hood and appropriately dispose of the generated solid and liquid wastes. Silver paint can cause skin irritation. You should avoid prolonged breathing of the vapor and dispose of the wastes appropriately.

SEM microscope training

Using an SEM microscope requires training. Contact your facility to learn how to use the microscope even before having a sample ready.

Alternative SEM sample preparation protocols

Multiple protocols for SEM on the inner ear have previously been developed, including the postfixation of the tissue with sequential incubations with osmium tetroxide (O) and thiocarbohydrazide (T), also known as OTOTO (Furness et al., 2008; Furness and Hackney, 1986; Heywood and Resnick, 1981). The OTOTO protocol has some advantages, such as fixing lipids and providing some electric conductivity to the sample. However, the OTOTO protocol preserves fewer links between the stereocilia and leads to more electrical charging when compared to the current procedure (see the limitations section for additional information).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
H_2O molecular grade	Corning	Cat#46-000-CM
Ethanol 200 proof, 99.5% 4 L	Acros Organics	Cat#61509-0040
Hepes buffer 1 M 20 mL	Gibco	Cat#15630-106
$MgCl_2$ 1 M	Sigma-Aldrich	Cat#63020-1L
$CaCl_2$ 1 M	Sigma-Aldrich	Cat#21114-1L
Sodium Chloride 3 kg	RPI research product	Cat#S23025-3000
D-Glucose	Thermo Scientific	Cat#AAA1682836
Aqueous Paraformaldehyde EM grade 32%, 10 × 10 mL vials[a]	Electron Microscopy Sciences	Cat#15714
Aqueous Glutaraldehyde EM grade 25%, 10 × 10 mL vials[a]	Electron Microscopy Sciences	Cat#16220
Other		
35 mm plastic dishes	CELLSTAR	Cat#627 160
1 mL syringe	BD Biosciences	Cat#329654
26G X $\frac{1}{2}$ (0.45 mm × 13 mm) needles	BD Biosciences	Cat#305111
Gloves	Genesee Scientific	Cat#44-100
Large Plastic Transfer Pipets Graduated	Globe Scientific	Cat#135040
Fine Plastic Transfer Pipettes	Fisher Scientific	Cat#13-711-6M
2.0 mL round bottom microfuge tubes	Fisher Scientific	Cat#22-010-091
50 mL plastic tubes	USA scientific	Cat#227261
Glass container 50 × 36	Kimax	Cat#23000
24 specimen critical point drying (CPD) sample holder	Tousimis	Cat#8763
Medium forceps (Dumont 5)	Fine Science Tools	Cat#11252-20
Fine forceps (Dumont 55)	Fine Science Tools	Cat#11255-20
Dissection scissors	Fine Science Tools	Cat#14090-09
Spoon or Abadie curette, 8 mm	Moria	Cat#1121B

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Micro dissecting spoon or curette, 5," size 3 2.5 mm	Biomedical Research Instruments	Cat#15-1025
Micro pick, straight	Ted Pella Inc	Cat#13532
SEM specimen mount gripper	Ted Pella Inc	Cat#1663-12
Carbon Conductive Tape, 12 mm W × 5 m L	Ted Pella Inc	Cat#16084-7
Conductive epoxy/silver paint[b]	Electron Microscopy Sciences	Cat#12642-14
Flat studs, 6 mm pin	Ted Pella Inc	Cat#16111
45° Beveled studs short 6 mm pin	Ted Pella Inc	Cat#16102-9
SEM Sample box	Ted Pella Inc	Cat#16160
Osmometer	Advanced Instruments	Cat#Model 3250
Stereomicroscope	Nikon	Cat#SMZ1000
Critical Point Drying	Tousimis	Cat#Autosamdri-815A
Sputter coater with deposit monitor	Electron Microscopy Sciences	Cat#150TS
Palladium target 57 mmm 0.1 nm thick	Electron Microscopy Sciences	Cat#Palladium
Iridium target 57 mmm 0.1 nm thick	Electron Microscopy Sciences	Cat#Iridium
Vertical auto-desiccator cabinet	SP Bel-Art	Cat#F42074-1115
Field emission source Scanning Electron Microscope	FEI	Cat#Magellan 400 XHR
Experimental models: Organisms/strains		
Mus Musculus C57BL/6J WT male and females P0-P11	JAX	Cat#000664

[a] Paraformaldehyde aqueous solution can cause skin irritation. Glutaraldehyde is more toxic, and you should avoid its inhalation. Open the glass vials containing the fixatives under a fume hood and dispose appropriately of the generated solid and liquid wastes.
[b] Silver paint wastes should also be disposed of appropriately.

MATERIALS AND EQUIPMENT

Solutions

Dissection buffer

Reagent	Final concentration	Amount
1 M Hepes buffer pH 7.2	0.05 mM	5 μL
1 M $CaCl_2$	2 mM	200 μL
1 M $MgCl_2$	0.5 mM	50 μL
NaCl powder	0.9% W/V	0.9 g
H_2O EM grade	n/a	90.74 mL
Total	n/a	100 mL

Adjust the osmolarity to 310 mOsm with D-glucose.
Store at 4°C and use for a week.

Mild fixative

Reagent	Final concentration	Amount
Aqueous Paraformaldehyde EM grade 32%	4%	6.25 mL
Dissection buffer	~1×	43.75 mL
Total	n/a	50 mL

Store at 4°C, use the same day, then dispose of the reagent appropriately.

Strong fixative

Reagent	Final concentration	Amount
Aqueous Paraformaldehyde EM grade 32%	4%	6.25 mL
Aqueous Glutaraldehyde EM grade 25%,	2.5%	5 mL
Dissection buffer	~1×	38.75 mL
Total	n/a	50 mL

Store at 4°C, use the same day, then dispose of the reagent appropriately.

Dehydration baths					
Reagents	15% ethanol	30% ethanol	50% ethanol	75% ethanol	95% ethanol
H_2O EM grade amount	42.5 mL	35 mL	25 mL	12.5 mL	2.5 mL
Ethanol 100% amount	7.5 mL	15 mL	25 mL	37.5 mL	47.5 mL
Total	50 mL	50 mL	50 mL	50 mL	50 mL

Store at 4°C and use for a week.

STEP-BY-STEP METHOD DETAILS

Step 1: Mouse inner ear extraction and fixation

⏱ Timing: 1 h for a single sample, count 30 min for each additional sample

The first step is to extract the inner ear from the animal skull. Next, mild fixation is performed by perfusing the cochlea to preserve the hair bundle structure without "gluing" the tectorial membrane onto it. The cochlea is then dissected, and the tectorial membrane removed cleanly, revealing the underlying hair bundles. The sample is then strongly fixed to increase its rigidity and resistance to mechanical damage during the remaining sample preparation and imaging.

1. The mouse is euthanized following laboratory animal care procedures, and then decapitated.
2. An incision is made with scissors to the skin from the back of the head, and the skin is peeled off the skull.
3. The skull is sectioned in two halves with scissors, and the brain tissue is removed with a thin spatula (Figure 2A).
4. The skull halves are cut with scissors to keep only the portion with the inner ear. The inner ear is visible at the inner surface of the temporal part of the skull. Blood vessels surrounding the inner ear are evident anatomical hallmarks that guide the cut (Figure 2B).

   ⚠ CRITICAL: If the cut is not done with care, the inner ear can be damaged. Learn this step under a stereomicroscope.

5. The skull pieces containing the inner ear are placed in a 35 mm plastic petri dish containing the dissection buffer at room temperature (RT) (Figure 2C).
6. The inner ear is detached from the skull using forceps by gently removing the connecting tissue between both structures (Figures 2D and 2E).
7. The inner ear is transferred to a new 35 mm dish with mild fixative (4% Paraformaldehyde) at RT (Figure 2F).

   ⚠ CRITICAL: The transfer of the inner ear tissue must always be performed in liquid. If the hair bundle is in contact with air, even momentarily, irreversible damage will occur. The tip links connecting stereocilia are particularly susceptible to this type of damage.

8. From this point, use the stereomicroscope.
9. Mesenchymal tissue is cleared from the cochlea, and the stapes ossicle is removed from the oval window.
10. A hole is made near the apex of the cochlea using a pick or a forceps (Figure 2G).
11. Fill a 1 mL syringe connected to a 26G needle with the mild fixative.
12. Place the syringe at the oval window and slowly perfuse 0.5 mL of mild fixative (Figure 2H; Methods video S1).
13. The fixative should be seen coming out of the apical hole (Figure 2H).
14. Place the needle at the round window and gently perfuse 0.5 mL of mild fixative (Figure 2I).
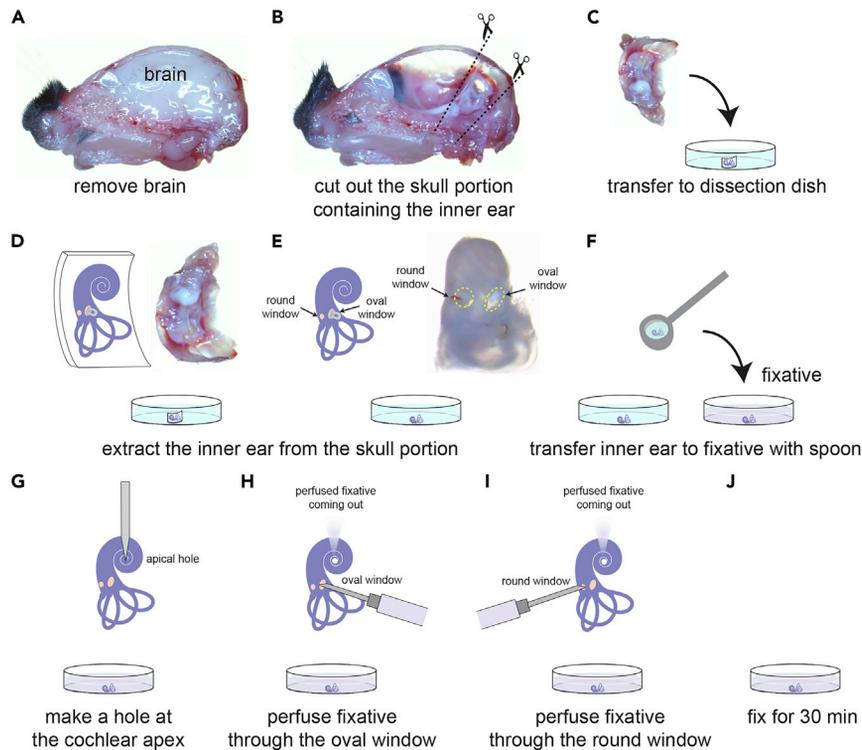
Figure 2. Inner ear extraction and mild fixation (steps 1–15)

(A) Hemi-section of a P11 mouse head with removed skin (steps 1–2).

(B and C) The brain is removed with a spatula, and the portion of the temporal part of the skull containing the inner ear is cut out and placed in a 35 mm dish with dissection buffer (steps 2–4). The blood vessels surrounding the inner ear can be used as a cutting landmark. Graphics of the inner eare adapted with permission from Shutterstock.

(D and E) The inner ear is detached from the skull (steps 5–6).

(F) Using a spoon, the inner ear is transferred to a 35 mm dish containing mild fixative, and the mesenchymal tissue and stapes ossicle are removed (step 7–9).

(G) Make a hole at the cochlear apex (step 10).

(H) Using a 1 mL syringe and a 35G needle, perfuse 0.5 mL of the mild fixative through the oval window. The fixative should be observed exiting the apical hole (steps 11–13).

(I) Repeat the step through the round window (step 14).

(J) Incubate in the fixative for 30 min without shaking and at RT (step 15).

⚠ CRITICAL: Stop the perfusion before any air bubbles come out of the syringe; otherwise, damage to the tissue will occur.

15. The inner ears are incubated in the fixative for 30 min at RT in the 35 mm petri dish without rocking (Figure 2J).

16. The inner ears are transferred with a spoon in the dissection buffer (Figure 3A).

17. With one forceps, grip the inner ear at the vestibule level.

⚠ CRITICAL: Depending on the age, the inner ear will vary in hardness as it progressively ossifies. Apply enough force to maintain control of the tissue without crushing it.

18. Using forceps with a crooked tip (e.g., damaged forceps), gently remove the cochlear bone like the shell of an egg (Figures 3B, 3B', 3C, and 3C'; Methods video S2).

⚠ CRITICAL: Again, the tissue will react differently depending on the animal's age dissected. At P0-P3, the structure is fragile; between P4-P7, it is more robust, and the dissection is
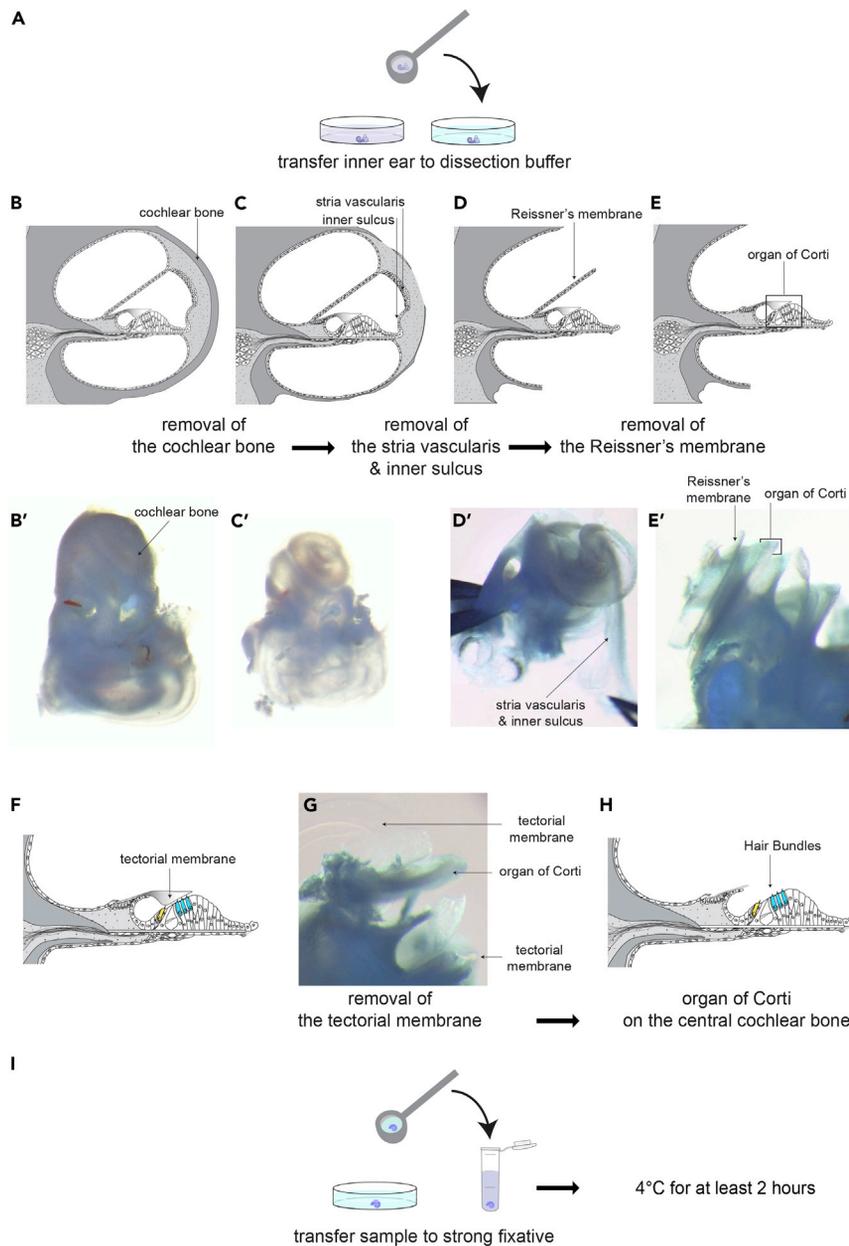
Figure 3. Organ of Corti dissection (steps 16–24)

(A) After fixation, the inner ear is transferred to a dish containing dissection buffer (step 16).

(B and B') A cartoon representing a cross-section of a cochlear turn before its dissection and picture of a P7 mouse inner ear before dissection (step 17).

(C and C') Cochlear bone is removed (step 18).

(D and D') Stria vascularis and inner sulcus are removed, pinching the structures at the base of the cochlea and unrolling them toward the apex (step 19).

(E and E') The Reissner's membrane is removed (step 20).

(F and G) The translucent tectorial membrane covers the hair cell hair bundles and is removed from the organ of Corti (step 21).

(H) The organ of Corti is free from the tectorial membrane and still attached to the central cochlear bone, which facilitates downstream sample manipulations (step 22).

(I) The dissected cochlea is transferred to a 2 mL round bottom tube containing strong fixative. The tube is kept a 4°C (without agitation) for at least 2 h (step 23–24).

easier. Starting from P8 onwards, the cochlea dissection becomes increasingly challenging with its ossification: pieces of cochlear bone could come out with the sensory epithelium. In P10 and older animals, the apical turn can consistently be dissected; it is more challenging for the rest of the cochlea.

19. The stria vascularis and inner sulcus are removed by pinching the structures at the cochlear base or apex and unrolling them up slowly until they detach from the cochlea (Figures 3C, 3C', 3D, and 3D'; Methods video S3).

    Note: For animals older than P7, the medial and basal part of the cochlea should be dissected differently: from the base, insert a forceps branch in the roof of the scala tympani and gently tear apart the tissue and progress apically. Skip step 20 (leave the Reisner's membrane in place; you will remove it after critical point drying).

20. The Reissner's membrane is removed (Figures 3D, 3D', 3E, and 3E'; Methods video S4).
21. The tectorial membrane (TM) is removed. Because of its translucence, the TM is difficult to visualize. Instead, gently pinch the forceps at the organ of the Corti surface either from the apical or the base. Once the TM is grabbed, it will detach easily and can be unrolled from the cochlea (Figures 3F–3H; Methods video S5). However, the TM is stickier at early postnatal age and may need to be removed in pieces.
22. The vestibule is removed from the cochlea by repeatedly puncturing the junctional area with a branch of forceps (Methods video S6). The organ of Corti can be left on the central cochlear bone (the modiolus), which facilitates the transfer procedure and better protects the hair bundle during the rest of the procedure. Alternatively, the organ of Corti can also be removed from the cochlear bone and processed (Methods video S7).
23. The dissected samples are then transferred with a smaller spoon to a 2 mL tube containing the strong fixative (2.5 % Glutaraldehyde, 4% Paraformaldehyde in dissection buffer) (Figure 3I).
24. The samples are placed at 4°C (without agitation) for the fixation, which should last a minimum of 2 h and can be extended to 15–20 h without adverse effects.

    ⏸️ Pause point: Once in the fixative, samples are safe, and can be synchronized.

    ⚠ CRITICAL: Appropriately dispose of the generated biological and chemical wastes (fixatives).

Step 2: Sample dehydration

🕐 Timing: 2 h 30 min

To prevent damage of biological samples when in the high vacuum of the SEM microscope chamber, the samples must be dehydrated. Otherwise, the water will exit the sample and damage its structure. First, optimal dehydration is reached by progressively replacing the aqueous solvent with ethanol, then replacing the ethanol with liquid CO_2. Finally, taking advantage of the low temperature and pressure at which liquid CO_2 becomes fluid, effuse it gently in a critical point drying (CPD) instrument.

25. After fixation, the samples are washed with dissection buffer (3 × 5 min). Use plastic Pasteur transfer pipettes to remove or fill solutions efficiently, but always leave some liquid in the bottom covering the samples to prevent tissue damage (Figure 4A).
26. During this time, prepare the ethanol dilutions in water (see next step) and cool them on ice.
27. The samples are then washed with water (2 × 5 min) and then with increasing percentages of ethanol diluted with water each time for 5 min and on ice without rocking:
    a. 15% ethanol
    b. 30% ethanol
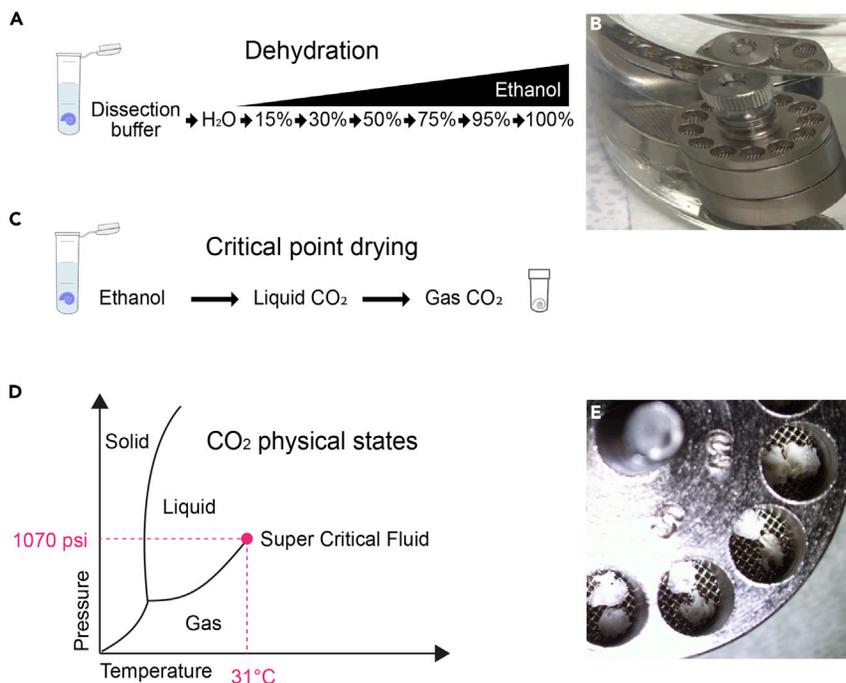
Figure 4. Sample dehydration and critical point drying (steps 25–34)

(A) After fixation, the samples are washed with dissection buffer (2 × 5 min) and then washed with ice-cold H_2O, 15%, 30%, 50%, 75%, 95%, and twice 100% ethanol for 5 min each and on ice (steps 25–27).

(B) The samples are transferred with a spoon to a critical point drying sample holder that has itself been submerged in a glass container filled with ice-cold 100% ethanol (steps 28–29).

(C and D) The critical point drying sample holder is placed in the chamber of a critical point drying instrument, pre-filled with 100% ethanol. The instrument will cool the ethanol, replace it with liquid CO_2, and reach the CO_2 critical point drying at 31°C and 1,070 psi. At this point, liquid CO_2 will come fluid and slowly effuse from the sample without damaging the sample structure (steps 30–33).

(E) After the procedure, the samples become white and highly fragile. The samples can be stored in a desiccator or directly mounted. The diameter of a well from this sample holder (2 × 12 wells) is 4 mm (step 34).

    c. 50% ethanol
    d. 75% ethanol
    e. 95% ethanol
    f. 100% ethanol
    g. 100% ethanol

28. Place the open CPD sample holder in a glass container filled with 100% ethanol (Figure 4B).
29. Transfer the dehydrated samples to the wells of the CPD sample holder using the spoon.

⚠ CRITICAL: The samples must stay in liquid at all times.

⚠ CRITICAL: Keep track of the location of each sample in the sample holder. Close the lid of the sample holder while it is in the ethanol.

⏸⏸ **Pause point:** The samples can be kept at 4°C at this stage (seal the glass container with parafilm)

Note: An alternative to CPD for dehydration is using low surface tension solvent hexamethyldisilazane (HMDS). HMDS is miscible in ethanol and used after ethanol dehydration. HMDS evaporates

from the tissue in less than an hour without special equipment. However, like stereocilia, the delicate cellular protrusions are distorted from the HMDS evaporation (Forge et al., 1992).

30. To use a CPD instrument, go to an EM facility with the samples.
31. The sample holder is quickly transferred from the glass container to the CPD instrument chamber, pre-filled with 100% ethanol.
32. The lid of the CPD chamber is tightly closed.
33. The CPD machine is started (follow instrument running instruction). The chamber will be cooled to $-20°C$, and liquid CO_2 will be injected into the CPD chamber, followed by a double purge to replace the ethanol with liquid CO_2 fully. The machine will then increase the pressure and the temperature of the chamber to reach the liquid CO_2 critical point, where the physical state of CO_2 is at the liquid/gas interface (Figures 4C and 4D). In this way, the liquid within the sample becomes fluid and is gently effused without damaging the sample. As a result, the sample is entirely dehydrated at the end of the run and becomes white (Figure 4E).
34. The samples can be stored in a desiccator or directly mounted.

⚠ CRITICAL: CPD samples are incredibly fragile and sensitive to electrostatic charges. Samples can "fly" away when attempting to hold them with forceps.

⏸ Pause point: Samples (still in the CPD sample holder) can be stored in a desiccator without adverse effect for a few months as long they stay dry.

Step 3: Sample mounting and metal coating

⏱ Timing: 30 min per sample and 15–20 h drying

Biological samples need to be made electrically conductive to produce good SEM images. This is critical for the experiment's success: if the sample is not conductive enough or improperly grounded, electrical charges will accumulate and be discharged cyclically, generating the "charging" artifact. This artifact prevents image acquisition and, when extreme, even sample observation.

35. The sample must be further dissected to minimize the distance between the tissue of interest and the metallic mounting stud.

⚠ CRITICAL: The CPD samples can be dissected with forceps but with extreme care: touching or grabbing the sample will damage it. A decision must be made about a part of the sample that is not critical; this area will become the holding part. The experimenter can also take advantage of the electrostatic sensitivity of the sample to "attach" it transiently to the forceps to transfer it.

Note: The final dissection of the tissue can also be done before the dehydration. However, the surrounding tissue will not protect the hair bundles from damage against tube walls, and smaller pieces can be lost during dehydrations steps.

36. Place a piece of double-sided carbon tape on a stud. This stud will be used to transiently attach a sample for final dissection so that it does not fly away.
37. Transfer the sample and gently deposit it onto the tape (Figures 5A and 5B).
38. Prepare a second stud where the sample will be mounted (Figure 5C).

Note: Two major types of studs can be used: flat ones or beveled ones. The choice depends on the type of image you are looking for. For example, it is best to use a flat stud for a top view of the hair bundle. However, if your goal is to image the hair bundle is inside/front view to see the tip links, then a 45° radius beveled stud is better.
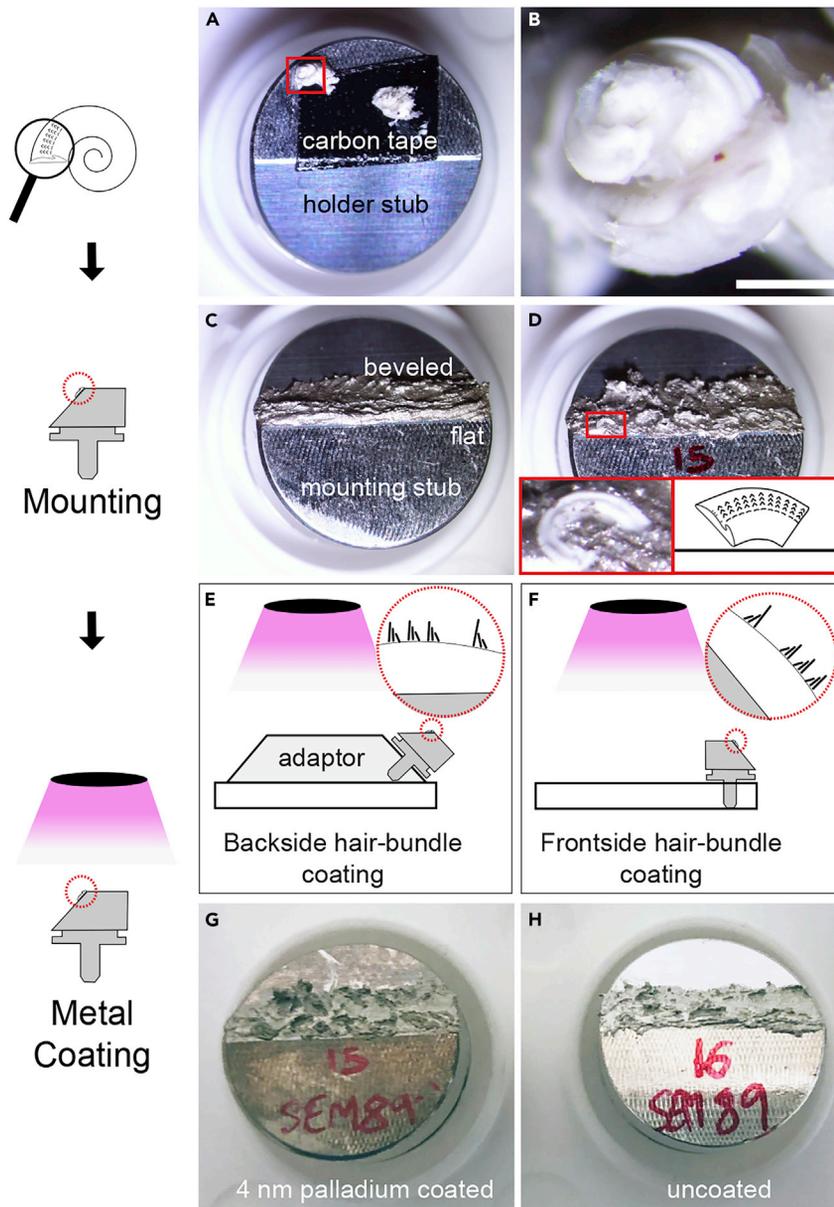
Figure 5. Dried-sample dissection, mounting on a stub, and metal coating (steps 35–46)

(A) The sample must be further dissected to minimize the distance between the tissue of interest and the metallic mounting stub. Because the samples are highly sensitive to electrostatic charges and can "fly" away easily, a sample is temporarily held in place on a double-sided carbon tape placed on a stub (a so-called holder stub) (steps 35–37).

(B) Dried apical turn of a P11 mouse cochlea (steps 35–37).

(C) A mixture of silver paint and epoxy is placed on another stub (the mounting stub). This stub has a 45° radius beveled face that is optimal for imaging the front side of hair bundles (steps 38–39).

(D) Parts of the cochlear turn are positioned tangentially to the stud middle edge (step 40).

(E) After drying 15–20 h in a desiccator, a thin layer of metal is deposited onto the sample. To coat all sides of the hair bundles and obtain optimal conductivity, the coating is first performed on the backside (using a custom-made adaptor) with 2 nm of Palladium (step 45a).

(F) Next, the front side of the hair bundles are coated with 2 nm of Palladium (steps 45b).

(G) A total of 4 nm of palladium had been coated onto the hair bundle with a sputter coater instrument (step 46).

(H) An uncoated sample for visual comparison with the coated sample in G (step 46). Stub diameter: 11 mm, Scale bar: B: 0.5 mm

39. Choose and prepare the mounting medium. Two types of mounting media can be used:
    a. The same double-sided carbon tape. It is the easiest way to mount samples but not always possible to use in the SEM microscope (see below).

    Note: While using the double-sided tape is convenient and easy, because of the tape's atomic composition, imaging will vaporize carbon atoms. This can affect the results of elemental analysis of surface experiments that other users of the SEM microscope might be doing. Confirm the compatibility of the mounting with the EM facility.

    b. A conductive silver paint and epoxy resin mixture at a 1:1 ratio using a pipet tip and a plastic petri dish. This mounting medium will not affect the elemental analysis of surface experiments.

    ⚠ CRITICAL: You need to test the appropriate time after mixing the two parts of the mounting medium: If used too early, the mixture may be too liquid and can penetrate your sample by capillarity and damage it. If used too late, the mixture could be too hard, and the electrical contact between the sample and the mixture will be too weak, increasing the risk of charging during imaging.

40. To obtain the best images, the working distance (the distance between the sample and the electron gun) must be short.
    a. For a flat stud, if the samples are to be imaged from the top, they can be distributed in the stud's entire surface. However, if a tilted-sample view is needed, the sample should be mounted along the perimeter edge of the flat stud, with the front side of hair cells facing outward. If the sample is too far from the edge, the working distance after tilting the stage might be too long to obtain a good resolution.
    b. For a beveled stud (with the goal of imaging the front side of the hair bundle), the sample must be placed at the edge between the beveled and the flat portions of the stud, with the hair bundles facing toward the flat surface of the stud.
41. To increase the number of hair bundles parallel to the central stud edge, it is recommended to "break" the cochlear turns into smaller parts with forceps and position them tangentially to the central edge. In addition, it is recommended to mount the samples in the cochlear apicobasal order (Figure 5D).
42. Once a stud is filled, draw a sample map on a sheet to reference it. This map will be handy to navigate between samples and link your results to a specific sample part.
43. For samples mounted with the conductive silver paint and epoxy resin, dry the sample 15–20 h by placing them in a desiccator.

    ⚠ CRITICAL: The sample must not contain liquid; otherwise, electric conductivity will be affected. The silver paint/epoxy resin mixture takes some time to dry. Do not shortcut it.

44. The sample needs to be coated with a metal layer to be electrically conductive. Choose the appropriate metal for your application.

    Note: The choice of the metal is based on the resolution you are interested in, the price, and the durability of the coating. Different metals produce different grain-sized, and some are more conductive than others. Historically, gold has been widely used because of its high conductivity, but its large grain-size (10–12 nm) is incompatible with fine surface details preservation. In our hands, iridium or palladium is the best option (see Table 1 and Heu et al., 2019 for more information).

45. Place your sample in the coating chamber of a sputter coater (likely found in your EM facility) with the appropriate metal target inserted.

Table 1. Sputter coating metal target choice for SEM

Target material	Grain size	Comments about use
Au (gold)	10–12 nm	For low magnification imaging.
Au/Pd alloy 80/20 (gold/Platinum)	4–8 nm	Not optimal for high-resolution imaging.
Pd (Palladium)	4 nm	Reduced charging effect and reduced secondary electrons production.
Pt (Platinum)	2–3 nm	Sensitive to oxygen, generating a "stress cracking" artifact to the sample.
Ir (Iridium)	1–2 nm	High SE production, charging effect frequently observed.
Cr (Chromium)	1–2 nm	Oxidizes in air contact, the sample must be imaged right away and will be damaged after.
W (Tungsten)	<1 nm	Oxidizes in air contact, the sample must be imaged right away and will be damaged after.

For more information, see (Heu et al., 2019).

46. The amount of metal to be coated needs to be determined: A thick coating will provide optimal electric conductivity but will affect the sample's surface; a too thin coating might still show charging artifacts. In our lab, we coat the sample in two steps to maximize the coverage of sample:
    a. First, the sample is placed onto a custom-made adaptor (made with epoxy putty found in home improvement store) for the 45°degree beveled stud samples to be used in the sputter coater: this compensates for the 45°degree, so the mounting surface is horizontal. Then, the samples are positioned away from the center so that the coating will mainly affect the backside of the hair bundles. About 2 nm of metal are sputter-coated this way (Figure 5E).
    b. Secondly, the sample is placed directly on the flat sample holder of the sputter coater, with the hair bundle facing the center of the holder. In this way, the front side of the hair bundle is coated with 2 nm of metal (Figure 5F).
47. The coated sample (Figures 5G and 5H) is then placed in a sample box and stored in a desiccator until observation.

⚠ CRITICAL: Samples are sensitive to water in the air. Always seal the sample box with parafilm when out of the desiccator. If the sample is protected from humidity, it can be reimaged for years. A fresh thin metal coating can be added to the sample to reimage it.

Step 4: SEM sample imaging and analysis

⏱ Timing: 3–5 h session

All the steps described above must have been correctly executed to produce good SEM images.

48. Reserve the microscope
49. Vent the SEM chamber and place your sample in the sample holder of the SEM stage.
50. Close the chamber and generate a vacuum.
51. Follow the standard procedure for plasma sample cleaning.
52. Proceed with the standard procedure of the SEM (beam centering, stage-height positioning, alignment of the condenser lens, and stigmatism).

⚠ CRITICAL: Aligning the microscope is critical to obtaining high-resolution images. It can take 45 min to perform alignment properly. The ETD (Everhart-Thornley Detector) detector is used for initial inspection of the sample and low mag imaging as it has a larger depth of field. The TLD (Through the Lens Detector) detector is recommended for high-resolution pictures, particularly in its "lens immersion mode." In this setting, sub-nanometer resolution can be reached. However, the depth of field is reduced. Alignment of the beam, lens, and stigmatism must be redone when the detector or the beam current is changed.

53. Identify the region of interest. If necessary, tilt the stage to be in the optimal viewing position.

> ⚠ CRITICAL: Be extremely careful when making this adjustment, so the sample does not touch and damage the electron gun.

54. Obtain the best focus on a hair bundle that is not critical. By doing so, you will determine the effect of the beam current on the structure.
55. Move to a nearby hair bundle, obtain a quick focus, zoom out to frame the entire hair bundle, adjust the image angle (scan rotation), and start the acquisition.

> *Note:* The standard imaging settings at the Grillet lab are obtained at 5 kV, 25 pA, 4 mm Working Distance, imaging 30 sec dwell timeline at 2048 × 1887 pixels (7.7 MB), which takes about 4 min.

56. Save and name the file with all information about the sample.

> ⚠ CRITICAL: Always shoot a low mag picture of your sample of interest for future reference and be able to assign higher resolution pictures to a position along the cochlea length.

57. Annotate your sample map to record the quality of the sample according to your goals.
58. End your SEM session by stopping the beam, bringing the stage to the loading position, and venting the chamber.
59. Remove the sample from the microscope, close the chamber, and start the pump to re-establish the vacuum.
60. Compare SEM pictures of each sample to find differences. Then, we recommend making some picture plates in Photoshop (Adobe), with all the sample pictures in one column.
61. If a difference is suspected, it needs to be quantified to ensure statistically significant and not just an experimental artifact.

EXPECTED OUTCOMES

Upon completion of this protocol, you should obtain crisp images of the hair bundle (Figures 6A–6C). Tip links are fragile structures but can easily be preserved and observed with this protocol (Figures 6D and 6E) (Miller et al., 2021; Trouillet et al., 2021).

LIMITATIONS

The main limitation of this protocol is that the plasma membrane is not preserved because of the lipid extraction that occurs during dehydration. Therefore, the plasma membrane can only be retained if pre-fixed with osmium tetroxide. However, osmium tetroxide alone or in the OTOTO protocol (see "before you begin" section) is more prone to charging and affects the stereociliary links (Figures 7A–7C).

TROUBLESHOOTING

Obtaining excellent results with this protocol requires diligence. Here are the main issues that you might encounter:

Problem 1

You fail in dissecting the organ of Corti (step 1–22).

Potential solution

Train with P7 inner ear repeatedly, as cochlear dissection takes some time to learn. Spend time recognizing each of the cochlear substructures under the microscope from the anatomy panels in Figure 1.
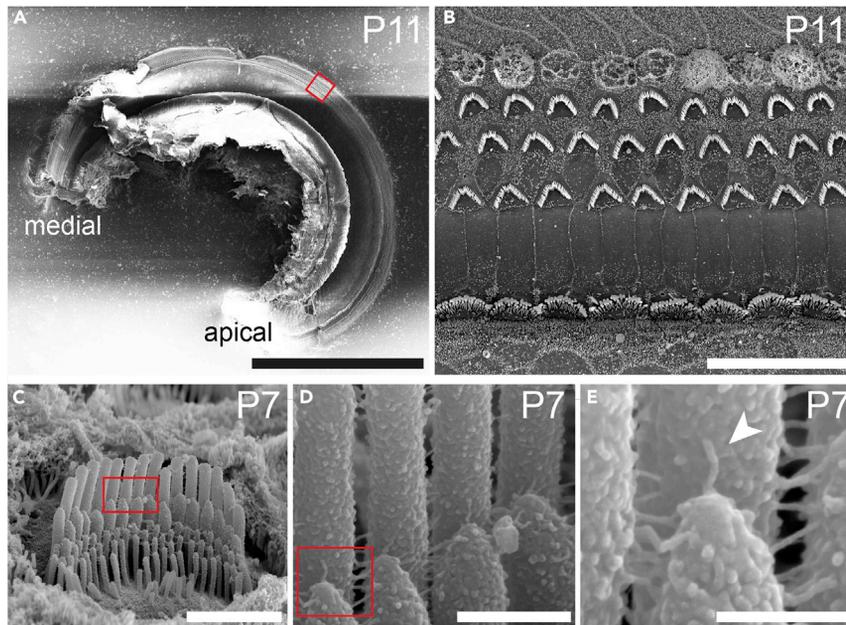
Figure 6. Examples of good SEM imaging of the cochlear hair bundles and tip links

Areas corresponding to zoomed-in pictures presented in the next panel are indicated with a red border square or rectangle.

(A) Top view of an apical-medial cochlear turn from P11 mouse imaged at low-resolution.

(B) Top view of a portion of the apical-medial cochlear turn with the hair bundles of inner hair cell and the three rows of outer hair cells visible.

(C) Front view of an inner hair cell hair bundle at P7.

(D) Higher-magnification showing the beveled-shaped second-row stereociliary tips and their filamentous extracellular connections to the neighboring stereocilia.

(E) Higher-magnification of the tip of a second stereociliary row connected to its taller neighbor by a tip-link (arrowhead), which transmits the force of stereocilia deflection to the mechanotransducer ion channels. Scale bars: A: 500 μm; B: 20 μm; C: 2 μm; D: 400 nm; E: 200 nm.

Problem 2

Upon SEM observation, the sample constantly changes contrast (Figure 7C).

Potential solution

This indicates that the sample is not adequately grounded or electrically conductive. The charging artifact can be addressed by adding more silver paint / epoxy to establish grounding or recoat the sample with an additional metal layer.

Problem 3

No hair bundle is observed on the sample by SEM imaging.

Potential solution

First, refer to the histology of the tissue to know where to expect to find the hair bundles. A classic error is mounting the cochlear turn upside down. To prevent, first note the direction of the spiral before dissecting the sample (clockwise or counterclockwise). Second, if you look at the sample from the side (slice view), a bump should be seen on the upper side, while the lower side is flat (Figures 3G and 3H). Finally, if the sample orientation is correct, but hair bundles are still not observed, another classic issue is that the tectorial membrane is still in place and is covering the hair bundles (Figure 7D). Unfortunately, the sample is useless when this occurs, and the experiment must be repeated.

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Protocol
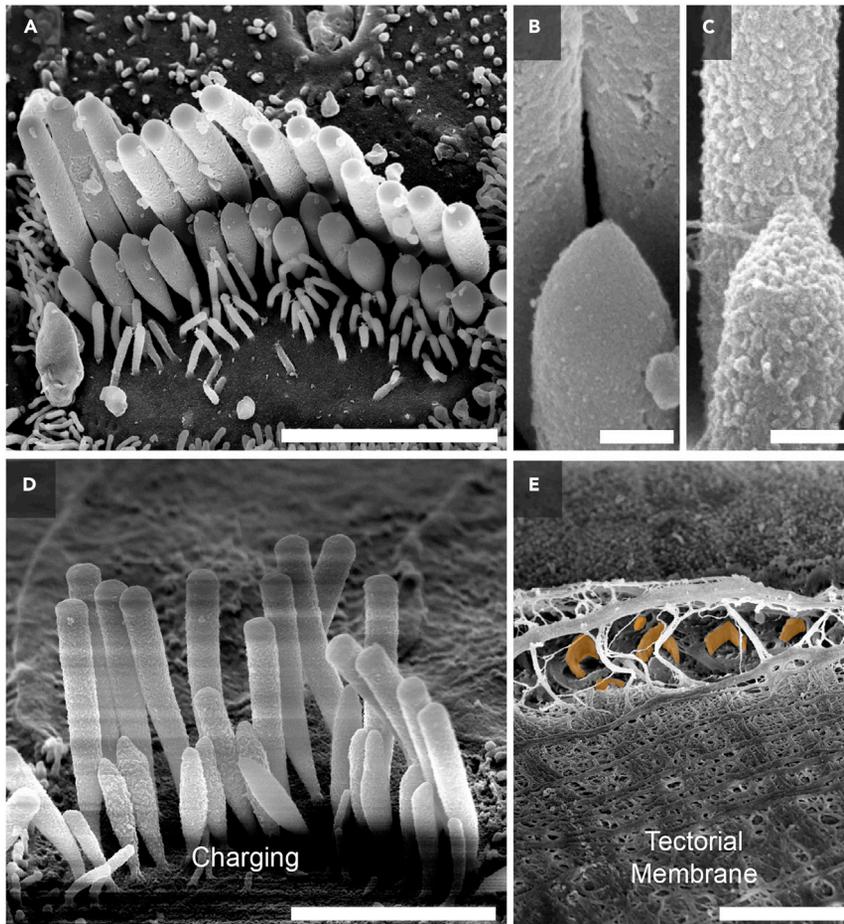
Figure 7. Limitations and troubleshooting of hair bundle SEM imaging

(A) Inner hair cell hair bundle prepared with the OTOTO (sequential osmium tetroxide and thiocarbohydrazide baths) postfixation. The majority of extracellular links have been removed.

(B) Higher-magnification of a second-row tip prepared with the OTOTO postfixation. Note the smooth surface of the stereocilia, which corresponds to the membrane.

(C) Higher-magnification of a second-row tip prepared with the protocol presented here. Note the rough surface of the stereocilia, which corresponds to the stereocilia without its membrane, and note the presence of the extracellular links.

(D) Example of a charging sample leading to a poor hair bundle SEM image.

(E) Example of a sample with the tectorial membrane still in place which covers the hair bundles (colored in oranges for outer hair cells). Scale bars: A: 3 μm; B: 200 nm; C: 200 nm; D: 2 μm; E: 10 μm.

Problem 4

The hair bundle and its stereocilia bend and cannot be imaged upon SEM observation.

Potential solution

This is an indication that the preparation is particularly fragile. This occurs when the sample retains liquid or encounters some humidity before being mounted and coated. This issue could potentially be addressed by using a lower electron beam current, imaging from a lower magnification, or imaging faster (at a lower resolution). If these solutions are insufficient, the sample can be recoated.

Problem 5

Upon SEM observation, the tip links are not observed.

Potential solution

The tip links are particularly fragile. If the sample had been in contact with air during transfer, the tip links could have been destroyed. Therefore, until P10, the tip links should be consistently observed; after P10, it is more challenging to preserve them.

RESOURCE AVAILABILITY

This study did not generate new unique reagents.

Lead contact

Further information should be directed to and fulfilled by the lead contact, Nicolas Grillet (ngrillet@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101213.

AUTHOR CONTRIBUTIONS

N.G. carried out methodology, investigation, and writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Forge, A., Nevill, G., Zajic, G., and Wright, A. (1992). Scanning electron microscopy of the mammalian organ of Corti: assessment of preparative procedures. Scanning Microsc. 6, 521–535.

Furness, D.N., and Hackney, C.M. (1986). High-resolution scanning-electron microscopy of stereocilia using the osmium-thiocarbohydrazide coating technique. Hear. Res. 21, 243–249. https://doi.org/10.1016/0378-5955(86)90222-4.

Furness, D.N., Katori, Y., Nirmal Kumar, B., and Hackney, C.M. (2008). The dimensions and structural attachments of tip links in mammalian cochlear hair cells and the effects of exposure to different levels of extracellular calcium. Neuroscience 154, 10–21.

Heu, R., Shahbazmohamadi, S., Yorston, J., and Capeder, P. (2019). Target material selection for sputter coating of SEM samples. Micros. Today 27, 32–36. https://doi.org/10.1017/s1551929519000610.

Heywood, P., and Resnick, S. (1981). Application of the thiocarbohydrazide-osmium coating technique to scanning electron microscopy of the inner ear.

Acta Otolaryngol. 91, 183–187. https://doi.org/10.3109/00016488109138498.

Miller, K.K., Atkinson, P., Mendoza, K.R., Maoiléidigh, D.Ó., and Grillet, N. (2021). Dimensions of a living cochlear hair bundle. Front. Cell Dev. Biol. 9, 3097. https://doi.org/10.3389/fcell.2021.742529.

Trouillet, A., Miller, K.K., George, S.S., Wang, P., Ali, N.-E.-S., Ricci, A., and Grillet, N. (2021). Loxhd1 mutations cause mechanotransduction defects in cochlear hair cells. J. Neurosci. 41, 3331–3343. https://doi.org/10.1523/jneurosci.0975-20.2021.