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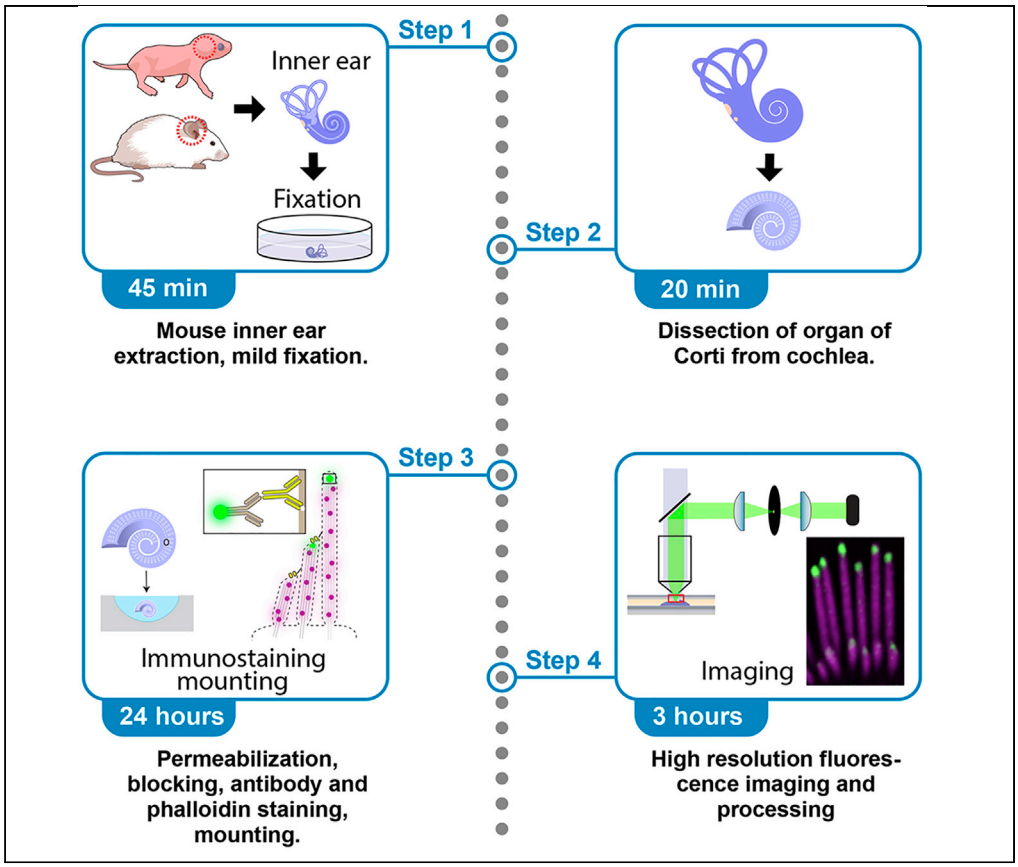
High-resolution immunofluorescence imaging of mouse cochlear hair bundles



High-resolution immunofluorescence imaging of cochlear hair bundles can be difficult because of the hair bundle's small dimensions, fragile nature, and complex organization. Here, we describe an optimized protocol for hair-bundle protein immunostaining and localization. We detail the steps and solutions for extracting and fixation of the mouse inner ear and the dissection of the organ of Corti. We note the optimal permeabilization, blocking, staining, and mounting conditions and the parameters for high-resolution microscopy imaging.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Katharine K. Miller, Pei Wang, Nicolas Grillet

kkmiller@stanford.edu (K.K.M.)
ngrillet@stanford.edu (N.G.)

Highlights

Techniques for dissecting the mouse cochlea and the organ of Corti

Dissection, permeabilization, blocking parameters to detect hair bundle proteins

Mounting method to localize protein in the hair bundles

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High-resolution immunofluorescence imaging of mouse cochlear hair bundles

Katharine K. Miller,[1,2,*] Pei Wang,[1] and Nicolas Grillet[1,3,*]

[1]Stanford University, Department of Otolaryngology — Head & Neck Surgery, Stanford, CA, USA

[2]Technical contact

[3]Lead contact

*Correspondence: kkmiller@stanford.edu (K.K.M.), ngrillet@stanford.edu (N.G.)
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SUMMARY

High-resolution immunofluorescence imaging of cochlear hair bundles faces many challenges due to the hair bundle's small dimensions, fragile nature, and complex organization. Here, we describe an optimized protocol for hair-bundle protein immunostaining and localization. We detail the steps and solutions for extracting and fixing the mouse inner ear and for dissecting the organ of Corti. We further emphasize the optimal permeabilization, blocking, staining, and mounting conditions as well as the parameters for high-resolution microscopy imaging.

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

BEFORE YOU BEGIN

Institutional permissions

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.

General experimental considerations

Before performing this protocol, it is vital to have a detailed understanding of the complex anatomy of the inner ear to properly dissect and mount the epithelium containing the hair cells for immunofluorescence imaging (For an overview, see Figure 1 and next note). To assess protein localization within a stereocilium, three requirements must be met: First, the stereocilia themselves must be fluorescently labeled. The mushroom toxin phalloidin conjugated to a fluorophore can be used to label the filamentous actin packed within each stereocilia. Second, the integrity of the stereociliary arrangement must be preserved throughout the procedure. This becomes increasingly difficult after the second postnatal week in mice as the number of extracellular filaments connecting the stereocilia is reduced. Third, the hair bundle must be lying flat in the same orientation that sound stimulation would deflect it for optimal visibility when imaging samples mounted under a coverslip. Below, we describe how to optimize for each of these steps.

> ***Note:*** At the apical surface of each inner and outer hair cell is a staircase assembly of stereocilia (actin-filled cellular protrusions), which make up the hair bundle (Tilney and Saunders, 1983). Cochlear stereocilia are typically a few microns tall, and less than half a micron wide (Miller et al., 2021). Each shorter stereocilium is connected to a taller stereocilium through a thin extracellular filament, the tip link (Figure 1H) (Pickles et al., 1984). The hair bundle deflection increases the tension on the tip links, which in turn increases the open probability of the mechanotransduction channels (Howard and Hudspeth, 1988; Ó Maoiléidigh and Ricci, 2019).

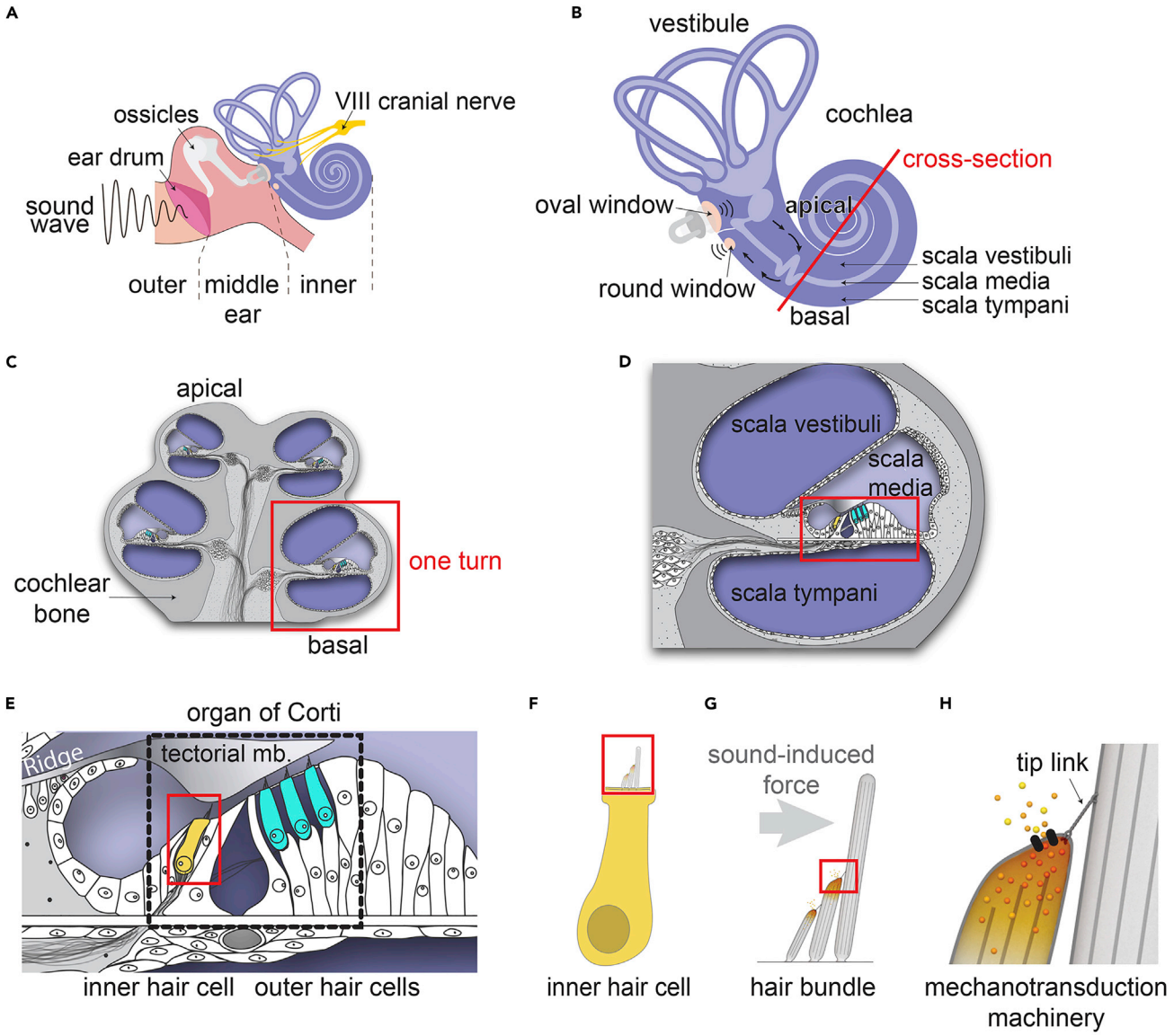

Figure 1. The process of hearing and the anatomy of the inner ear

(A) Sound waves pass through the outer ear, deflecting the ear drum, and passing vibrations through the three tiny ossicles before reaching the inner ear.

(B) The last ossicle – the stirrup-shaped stapes – is inserted into the oval window of the cochlea—the organ of hearing located within the inner ear. The vibrations generated by sound pass from the stapes through the oval window and travel through the turns of the cochlea.

(C and D) (C) A cross-section of the cochlea and (D) a close-up of a single turn cross-section reveal the organ of Corti spiraling through the cochlea.

(E) The organ of Corti contains the mechanosensitive hair cells, which lie beneath the tectorial membrane. The outer hair cells are the amplifier cells, whereas the inner hair cells electrically transmit mechanical inputs to the brain. The upper surface of the epithelium close to the modiolus forms a ridge, while the bottom part is flat.

(F) Hair bundles composed of actin-filled stereocilia arranged in a staircase configuration sit on top of the apical surface of the hair cells.

(G and H) (G) Sound-induced forces deflect the hair bundles, (H) putting tension on the thin tip link that connects the shorter stereocilia to their taller paired stereocilia. This tension increases the open probability of the mechanotransduction channel, allowing an influx of cations and depolarization of the cell. Graphics of the inner ear were adapted with permission from Shutterstock.

Many proteins are involved in the hair bundle's ability to transduce mechanical forces into electrical signals (Cunningham et al., 2020; Giese et al., 2017; Kawashima et al., 2011; Kurima et al., 2003; Xiong et al., 2012; Zhao et al., 2014). The distribution of these proteins in the hair bundle provides insight into their potential molecular roles. For example, a protein localized

at the tip of a transducing stereocilium could participate in the channel complex or its interaction with the cytoskeleton. Alternatively, a protein clustered on the side of a taller stereocilium slightly above the height of its shorter, paired stereocilium may play a role in anchoring the tip-link or regulating its tension.

Dissection conditions prior to fixation

Preserving the integrity of the organ of Corti tissue prior to fixation is of particular importance for hair bundle immunostaining, as the bundle protrudes from the apical surface of the hair cells and is vulnerable. Once the inner ear has been extracted from the temporal bone, it should quickly be placed in dissection medium with adjusted pH, Mg^{2+} and Ca^{2+}, and with the osmolarity of the solution at physiological levels.

For our protocol, we use Hanks Balanced Salt Solution (HBSS) to maintain a physiological pH and to keep the tissue alive outside of a cell incubator for a short period of time. We then add Mg^{2+} to help maintain tissue viability and Ca^{2+} to prevent the tip links from breaking during dissection (Assad et al., 1991). Although the precise mechanism of how Mg^{2+} helps to maintain tissue viability is unknown, Ca^{2+} is required for tip link integrity (Furness et al., 2008; Sendowski et al., 2011).

Finally, we adjust the osmolarity of our dissection medium to physiological levels, which results in healthier cells and tissue prior to fixation by preventing excess swelling or shrinkage that could affect immunostaining results. If an osmometer is not available, Leibovitz L-15 Medium can be used as an alternative. This medium comes pre-adjusted to physiological osmolarity levels; however, most providers report a range of osmolarity for their mediums. This could cause variability in the final samples.

Fixation conditions

Selecting the proper fixative for an experiment is a balance between properly preserving the tissue structure and maintaining the antigenicity of the tissue. There are many different chemical fixatives commonly used to prepare tissue, each with pros and cons. Some of the most common are listed below:

Fixative	Fixation process	Good for hair bundle immunostaining?
Formaldehyde/Paraformaldehyde (single aldehyde group)	Protein crosslinking via primary amines and sulfhydryl groups	Mild fixation preserves structure with generally a minimal impact on antigenicity
Glutaraldehyde (two aldehyde groups)	Dual protein crosslinking via primary amines and sulfhydryl groups	Causes autofluorescence in tissue; strong fixation generally affects antigenicity.
Methanol	Protein dehydration and precipitation	Can denature proteins; may interfere with phalloidin staining

Throughout this protocol, we use paraformaldehyde, or PFA, because the purchased chemical used is 32% aqueous paraformaldehyde. PFA permeates tissue slowly (1 mm per hour) and crosslinks proteins. It continues to fix the tissue over a long period of time (days to weeks). Short fixation – in our protocol, 30 min at 25°C – using PFA is enough to preserve the tissue structure with a minimal effect on tissue antigenicity. Longer fixations such as 24 h at 4°C or longer should be performed with care, as these may disrupt the ability for antibodies to recognize tissue antigens.

Glutaraldehyde is a much stronger fixative than PFA and is frequently used for electron microscopy preparations. Because of its large molecular dimensions compared to PFA, the tissue penetration rate of glutaraldehyde is much slower. Also, as a strong protein crosslinker, it is more likely than PFA to alter tissue antigenicity. Finally, it causes autofluorescence in tissue, which must be quenched prior to performing fluorescence microscopy by reducing the aldehyde group to an hydroxyl group with sodium borohydride or glycine.

Methanol fixation can be performed for some fluorescence microscopy protocols, and permeabilizes the tissue in addition to fixing it. However, it has the possibility to denature proteins and can interfere with phalloidin staining, so it should not be the first choice for hair bundle immunofluorescence.

> ***Note:*** If antibodies are being designed against a protein for use with PFA fixation, choose an antigen that does not contain a lysine residue, as they are crosslinked by aldehydes and are likely to reduce antigenicity (Vani et al., 2006).

Primary antibody validation

Because of its large surface area and glycosylation, the hair bundle is prone to nonspecific binding. Therefore, the specificity of primary antibodies must be thoroughly validated prior to hair bundle immunostaining.

The best validation of an antibody is provided by comparing the endogenous protein staining in a hair bundle from a wild-type (WT) mouse with that of a mouse model in which the coding sequence of the protein antigen has been inactivated. Alternatively, *in vitro* validation can be performed; this consists of overexpressing a tagged version of the protein of interest in a cell line and colocalizing the primary antibody with an anti-tag antibody. However, overexpression in cell culture generates an artificial immunostaining situation, where the ability to detect the large amounts of overexpressed protein may not translate to the low level of endogenous antigen. Furthermore, staining caused by cross-reaction with other proteins or nonspecific binding are likely different between the hair bundle and a cell line.

Another approach to support the validity of the antibody is to replicate the cellular specificity data obtained by previous immunostaining or mRNA expression (e.g., other antibodies, in situ hybridization, single-cell transcription) with your experimental results. Otherwise, a different tissue, in which a strong expression of the gene or protein of interest has previously been identified, can also be used as a positive control, and can provide more confidence in the specificity of the antibody.

Finally, if no primary antibody is available, the tagged cDNA of a given gene can be overexpressed by means of transgenic mice, injectoporation, or viral transduction of hair cells (Verdoodt et al., 2021; Xiong et al., 2012). The protein is then localized *in vivo* with an antibody directed against the tag and the result is compared to non-overexpressing hair bundles as a negative control. A limitation of this approach is inherent to protein overexpression in tissue, which can lead to artifactual localization.

Optimizing immunolabeling

To obtain the best staining and reduce background signal, the lowest concentration of primary antibody that produces a robust and specific staining should be used. It is also recommended to include a primary antibody with previously published specificity results as a positive control, to ensure the proper execution of the protocol.

The length of time and temperature of primary antibody incubation can also be modified for different protocols/antibodies. We have found that at least 12 h of incubation with primary antibody at 4°C usually gives us the best signal with the lowest background. However, other protocols use 25°C or 37°C, for 1–12 h, although these temperatures may reduce the signal-to-noise ratio.

When using fluorescently labeled antibodies, it is important to use fluorophores with significantly distant emission wavelengths to reduce the bleed-through between channels. Performing a negative control experiment without one of the fluorophores may be required to confirm the absence of bleed-through. Because commercial secondary antibodies have a high affinity, they should be used at a low concentration to increase the signal-to-noise ratio.

For extremely low-expressing proteins, unconjugated secondary antibodies can be used followed by tertiary antibodies. As multiple secondary antibodies can bind to each primary antibody, and multiple tertiary antibodies can bind to each secondary antibody, the signal from low levels of protein can be amplified.

Permeabilization conditions

Tissue permeabilization allows antibodies to penetrate the plasma membrane and bind to intracellular epitopes. Because the integrity of the stereociliary membrane may be necessary to localize certain proteins, it can be critical to preserve it. Therefore, pilot experiments are necessary to optimize permeabilization conditions.

The most commonly used detergents for hair bundle immunofluorescence are:

Detergent	Strength	Selectivity	Mode of permeabilization
Triton X-100	harsh	nonselective; extracts both lipids and proteins	inserts into the lipid membrane; irreversibly permeabilizes the membrane
Tween-20	milder	nonselective	permeabilizes membranes allowing antibodies access to the cytoplasm
Saponin	mildest	selectively interacts with and extracts cholesterols	reversible; does not permeabilize the nuclear membrane

For new immunostaining protocols, a range of detergent concentrations (0.01%–0.5%) should be tested.

Here, we use 0.05% Triton X-100 in the permeabilization step and 0.05% Tween-20 in all following steps (For more information, see "materials and equipment" section). Triton X-100 is used to perforate the plasma membrane, while Tween-20 is used to reduce surface tension forces and nonspecific binding. We have found that this combination works well for proteins located within or just below the stereociliary membrane, as many of the mechanotransduction-associated proteins are.

Blocking conditions

Properly blocking tissue before and during antibody incubation can significantly improve the quality of the immunostaining. Antibodies can improperly and nonspecifically bind to many different "sticky" antigens within the tissue. Blocking creates a competition for binding with these sites. Instead of the antibodies binding the antigens, the blocking compound binds to the "sticky" sites and prevents nonspecific antibody interactions. Blocking can be performed using a purified protein, such as bovine serum albumin (BSA), a serum from the animal species from which the secondary antibody has been produced, or a commercial blocking solution. Depending on the strength and specificity of the chosen antibodies, different blocking protocols may work better. The present protocol uses 4% BSA for initial blocking and 1% BSA in all following steps.

Mounting conditions

Prior to mounting your sample, you must consider the conditions of your downstream imaging. First, it is important to match the refractive index of the mounting components to achieve high resolution images. Glass coverslips have a refractive index of around 1.51, as does typical immersion oil. Making sure that the mounting medium has a refractive index close to that of the other mounting materials reduces light scattering and improves image quality. Non-hardening mounting medium stays in liquid form, and the refractive index will not change over time. This allows for immediate imaging after the coverslip is sealed. Hardening mounting medium needs time to cure to reach optimal refractive index.

The mounting strategy that we describe in this protocol brings the hair bundles very close to the glass coverslip, which reduces the effect of the mounting medium on the overall refractive index

for bundle imaging. In this case, imaging can be performed immediately after mounting, even with uncured mounting medium, with very good results. Still, allowing the mounting medium to cure may increase the quality of the hair bundle images.

Some mounting mediums have additional characteristics, such as the inclusion of DAPI to directly stain DNA and thereby localize the cell nuclei, or antifade to help extend the lifetime of the fluorescence by preventing photobleaching. This protocol uses Prolong Gold Antifade Mounting medium, which gives us excellent fluorescence results.

Finally, the thickness of the coverslip should be matched to what is labeled on the microscope lens you intend to use. Some objectives also have correction collars that allow adjustments to be made to match the coverslip thickness. It is best to check the lens you will be using before mounting your samples to know what coverslip to use. The microscope objective lenses that we use require 0.17 mm thick coverslips. Therefore, we use a #1$^1/_2$ high-performance cover glass, which has a thickness of 0.165–0.175 mm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
HBSS (1×), no $CaCl_2$, no $MgCl_2$, no $MgSO_4$	Gibco	Cat#14175-095
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
Bovine Serum Albumin (Fraction V) Heat-shock Treated	Fisher Scientific	Cat#BP1600-100
Glucose	Thermo Scientific	Cat#AAA1682836
Aqueous Paraformaldehyde EM Grade 32%, 10 × 10 mL vials*	Electron Microscopy Sciences	Cat#15714
Triton X-100	Sigma-Aldrich	Cat#T9284
Tween-20, 100% Nonionic Detergent	Bio-Rad	Cat#1706531
Phosphate Buffered Saline	Caisson Labs	Cat#PBL06
Alexa Fluor 568 Phalloidin** (1:500 dilution if methanol reconstituted, 1:1000 dilution if DMSO reconstituted)	Invitrogen	Cat#A12380
Methanol, 99.9%, for analysis	Thermo Fisher Scientific	Cat#AC176840010
Dimethyl Sulfoxide (DMSO), Fisher BioReagents	Thermo Fisher Scientific	Cat#BP231-1-100
Purified Mouse Anti-Espin, Clone 31/Espin (RU0); (1:200 dilution)	BD Biosciences	Cat#611656
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488; 1:500 dilution	Thermo Fisher Scientific	Cat#A-21206
Software and algorithms		
Imaris Microscopy Image Analysis Software	Oxford Instruments	https://imaris.oxinst.com/
Fiji	Open source	https://fiji.sc/
Other		
35 mm Plastic Dishes	CELLSTAR	Cat#627 160
1 mL Syringes	BD Biosciences	Cat#329654
26G × $^1/_2$ (0.45 × 13 mm) Needles	BD Biosciences	Cat#305111
Gloves	Genesee Scientific	Cat#44-100
15 mL Plastic Tubes	USA Scientific	Cat#1475-1611
50 mL Plastic Tubes	USA Scientific	Cat#227261
1000 µL Graduated Tips	USA Scientific	Cat#1111-2721
200 µL Graduated Tips	USA Scientific	Cat#1111-1700
Weighing Paper	Fisher Scientific	Cat#09-898-12A
Feather Scalpel Blades, #11	Electron Microscopy Sciences	Cat#72044-11

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
2.0 mL Low Adhesion Microcentrifuge Tubes, with Caps	USA Scientific	Cat#1420-2600
Foil Roll, Aluminum, Standard, 1,000 ft Roll Length, 12 in Width, Silver	Grainger	Cat#6CHG0
Premium Microscope Slides Superfrost	Fisher Scientific	Cat#12-544-7
Cover Glasses, High-performance, Thickness no. 1 $^1/_2$	Carl Zeiss	Cat#474030-9000-000
Prolong Gold Antifade	Invitrogen	Cat#P36934
Kimwipes	Kimberly-Clark	Cat#34155
Nail Polish	n/a	n/a
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
Micro Dissecting Spoon or Curette, 5″ Size 3 2.5 mm	Biomedical Research Instruments	Cat#15-1020
PIPETMAN Classic P1000	Gilson	Cat#F123602
PIPETMAN Classic P200	Gilson	Cat#F123601
PIPETMAN Classic P20	Gilson	Cat#F123600
Polypropylene Microtube Storage Racks	Fisher Scientific	Cat#05-541-1
Western Blot Box (for sample incubation)	MilliporeSigma	Cat#Z742091
Micro-Test Staining Dish	Electron Microscopy Sciences	Cat#71564
Osmometer	Advanced Instruments	Cat#3250
Stereomicroscope	Nikon	Cat#SMZ1000
Precision Balance	Mettler Toledo	Cat#MS802S/03
Multi-Platform Shaker	Fisher Scientific	Cat#13687700
LSM 880 (or equivalent)	Carl Zeiss	Cat#LSM880
4°C Room/Large Fridge	Various	Various
Experimental models: Organisms/strains		
Mus Musculus C57BL/6J WT male and females P6, P7, P11, and P21	JAX	Cat#000664

*CRITICAL: When working with paraformaldehyde (PFA), wear suitable personal protective equipment and, when possible, work in a hood. Dispose of PFA appropriately.
**Note: When co-staining with antibodies, make sure to select appropriate fluorophore for phalloidin.

MATERIALS AND EQUIPMENT

Dissection Buffer

Reagent	Final concentration	Amount
HBSS, No Mg^{2+} No Ca^{2+}	n/a	500 mL
1 M HEPES Buffer pH 7.2	0.05 mM	25 µL
1 M $CaCl_2$	2 mM	1 mL
1 M $MgCl_2$	0.5 mM	250 µL
Total	**n/a**	**500 mL**

Using an osmometer, adjust the osmolarity to 310 mOsm with D-glucose.
Store at 4°C and use within a week.

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 appropriately.

⚠ CRITICAL: When working with paraformaldehyde (PFA), wear suitable personal protective equipment and, when possible, work in a hood. Dispose of PFA appropriately.

Note: PFA could also be diluted in 1× PBS with Mg^{2+} and Ca^{2+} added.

Permeabilization Buffer		
Reagent	Final concentration	Amount
Bovine Serum Albumin	4%	0.4 g
PBS (Phosphate Buffer Saline)	1×	Up to 10 mL
Triton X-100	0.05%	5 µL
Total	**n/a**	**10 mL**
Store at 4°C, use the same day then dispose appropriately.		

Blocking Buffer		
Reagent	Final concentration	Amount
Bovine Serum Albumin	4%	0.4 g
PBS (Phosphate Buffer Saline)	1×	Up to 10 mL
Tween-20	0.05%	5 µL
Total	**n/a**	**10 mL**
Store at 4°C, use the same day then dispose appropriately.		

Dilution Buffer		
Reagent	Final concentration	Amount
Bovine Serum Albumin	1%	0.1 g
PBS (Phosphate Buffer Saline)	1×	Up to 10 mL
Tween-20	0.05%	5 µL
Total	**n/a**	**10 mL**
Store at 4°C, use the same day then dispose appropriately.		

STEP-BY-STEP METHOD DETAILS

Step 1: Dissection and fixation of the cochlea

Timing: 45 min

Because of the fragile nature of the hair bundle, dissecting and fixing the cochlea properly greatly increases the chances of obtaining properly mounted hair bundles for clear imaging.

1. The mouse is euthanized following laboratory animal care procedures and then decapitated.
2. Using dissection scissors, remove the skin of the head by cutting from the posterior, caudal base of the skull toward the rostral part of the skull.
3. Holding the front portion of the skull, hemisect the skull along the sagittal plane with dissection scissors (for younger mice) or a scalpel (for older mice) (Figure 2A).
4. Discard the hemisected brain and remove the portion of the temporal bone of the skull containing the cochlea and vestibule (Figure 2B).
5. Immediately place the bone containing the cochlea and vestibule into 25°C dissection buffer in a 35-mm dish (Figure 2C).

⚠ CRITICAL: Make sure that the osmolarity of the dissection buffer is at physiological levels as it can affect the tissue integrity.

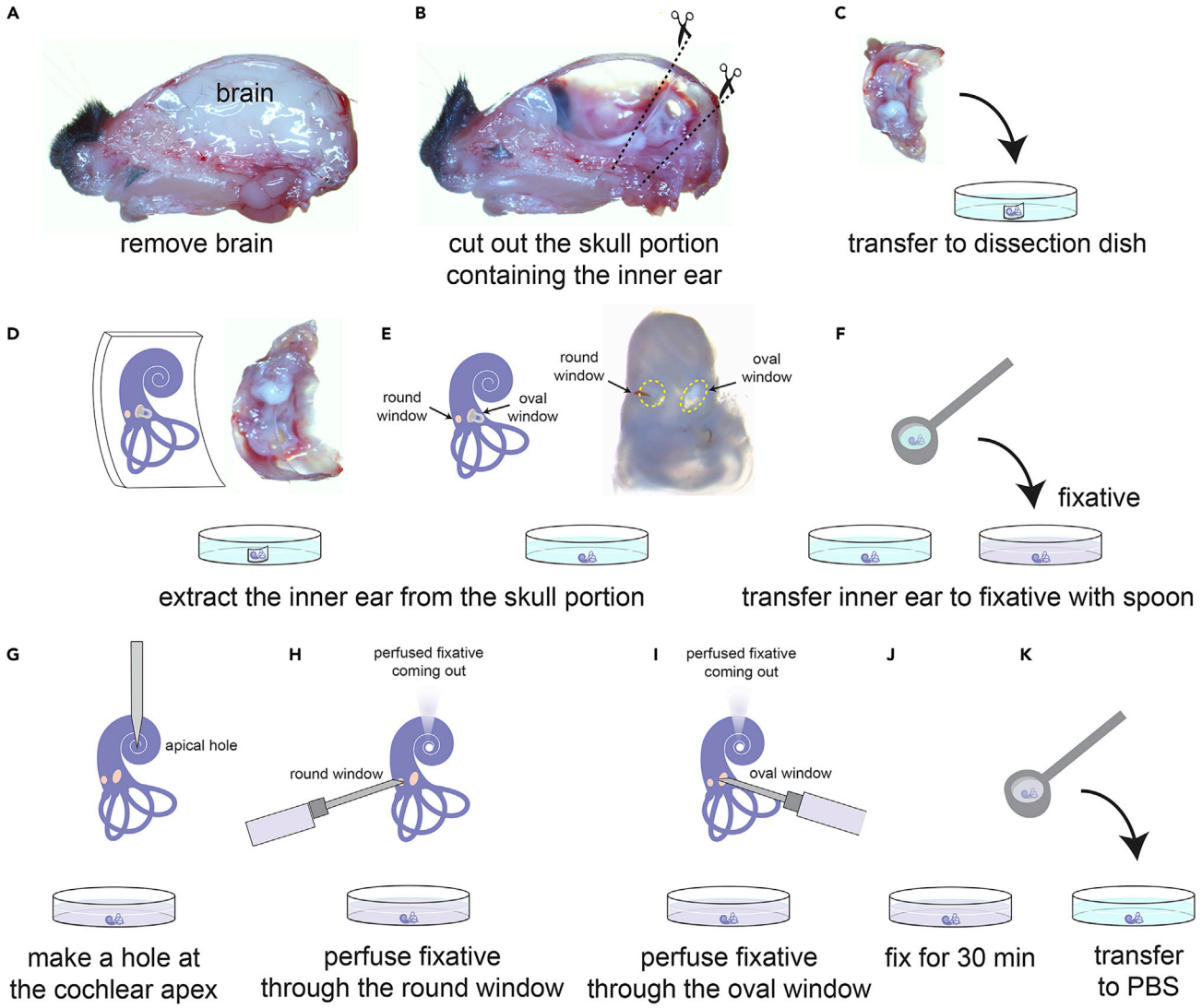

Figure 2. Initial dissection of the inner ear

(A) After hemisecting the skull, the brain is removed.

(B and C) (B) The portion of the skull containing the inner ear is extracted and (C) transferred to a dissection dish containing dissection medium.

(D) The inner ear is extracted from the surrounding bone.

(E) The stapes is removed from the oval window (if still present).

(F) The inner ear is transferred to a plate containing 4% PFA fixative using a small spoon.

(G–I) (G) A hole is made at the apex of the cochlea and (H) fixative is perfused through the round and (I) oval windows using a needle and syringe.

(J and K) (J) The cochlea is incubated in fixative for 30 min before (K) being transferred to PBS. Graphics of the inner ear were adapted with permission from Shutterstock.

Note: The dissection buffer is stored at 4°C once opened and used at 25°C for the procedure.

For a demonstration of steps 6 and 7, see Methods video S1.

6. Under the stereoscope, gently detach along the outer edges of the cochlea and vestibule with #5 forceps to extract the organs from the surrounding bone (Figure 2D).
7. Trim away additional tissue and (if still attached) remove the stapes from the oval window (Figure 2E).
8. Using a small spoon, transfer the cochlea to a 35-mm dish containing 4% PFA diluted in dissection buffer at 25°C (Figure 2F).

⚠ CRITICAL: During dissection and in all steps, great care must be provided to ensure that samples always stay immersed in liquid. This is particularly important for the maintenance of the tip links, as even brief moments in contact with air can cause them to break. (See troubleshooting section).

Note: For mice younger than P5, it may be possible to dissect the full organ of Corti very quickly and immerse it directly in fixative, rather than perfusing through the cochlea as described in steps 9 and 10. If a fast dissection is performed, skip ahead to step 11.

For a demonstration of steps 9 and 10, see Methods video S2.

9. Open a small hole in the apex of the cochlea (Figure 2G).
10. Using a 1-mL syringe with a 26G × $^{1}/_{2}$ needle, gently flush 0.5 mL fixative (25°C) through the oval window, and 0.5 mL fixative through the round window (Figures 2H and 2I).

Note: A flow of fixative out of the hole in the apex of the cochlea should be visible. Fixative is perfused at a rate of approximately 0.1 mL/sec.

11. Using a small spoon, transfer the cochlea to a 2-mL tube containing 1.5 mL of fixative.
12. Incubate in fixative at 25°C for 30 min (Figure 2J).
13. Using a spoon, transfer the fixed cochlea to a 2-mL tube containing 1.5 mL of PBS (Figure 2K).

Pause point: It is best practice to perform the immunostaining right after the tissue fixation. It is possible to store the tissue in PBS for a maximum of one to two days; however, this might affect the antigenicity.

Step 2: Fine dissection of the organ of Corti

Timing: 10–20 min depending on the mouse age

Excising the organ of Corti from the cochlear bone and removing the tectorial membrane, Reissner's membrane, and the stria vascularis allows the whole tissue to be mounted for clear imaging of the hair bundle.

For a demonstration of steps 14–19 at postnatal day 6, see Methods video S3.

For a demonstration of steps 14–19 at postnatal day 21, see Methods video S4.

14. Place the inner ear in a dish containing PBS (Figure 3A).
15. Starting at the hole in the apex of the cochlea, gently remove the outer bone along the cochlear length with #5 forceps (Figures 3B and 3C).
    a. Crack along the center of the spirals, avoiding the areas where the stria abuts the bone.
    b. Once a hole has been opened along the cochlea, slowly chip away at the remaining bone to reveal the stria.

Note: If the vestibule is still connected to the cochlea, it is an ideal place to grip with forceps to restrain the organ for dissection.

16. Again, starting at the apical point, slowly remove the stria vascularis (the outermost tissue forming a partial wall around the organ of Corti) using fine #55 forceps.

Note: For P6 and younger mice, the whole stria can often easily be removed in one attempt, spiraling down the organ. For older mice, extreme care must be taken in trimming below the apical region (Figures 3C and 3D).

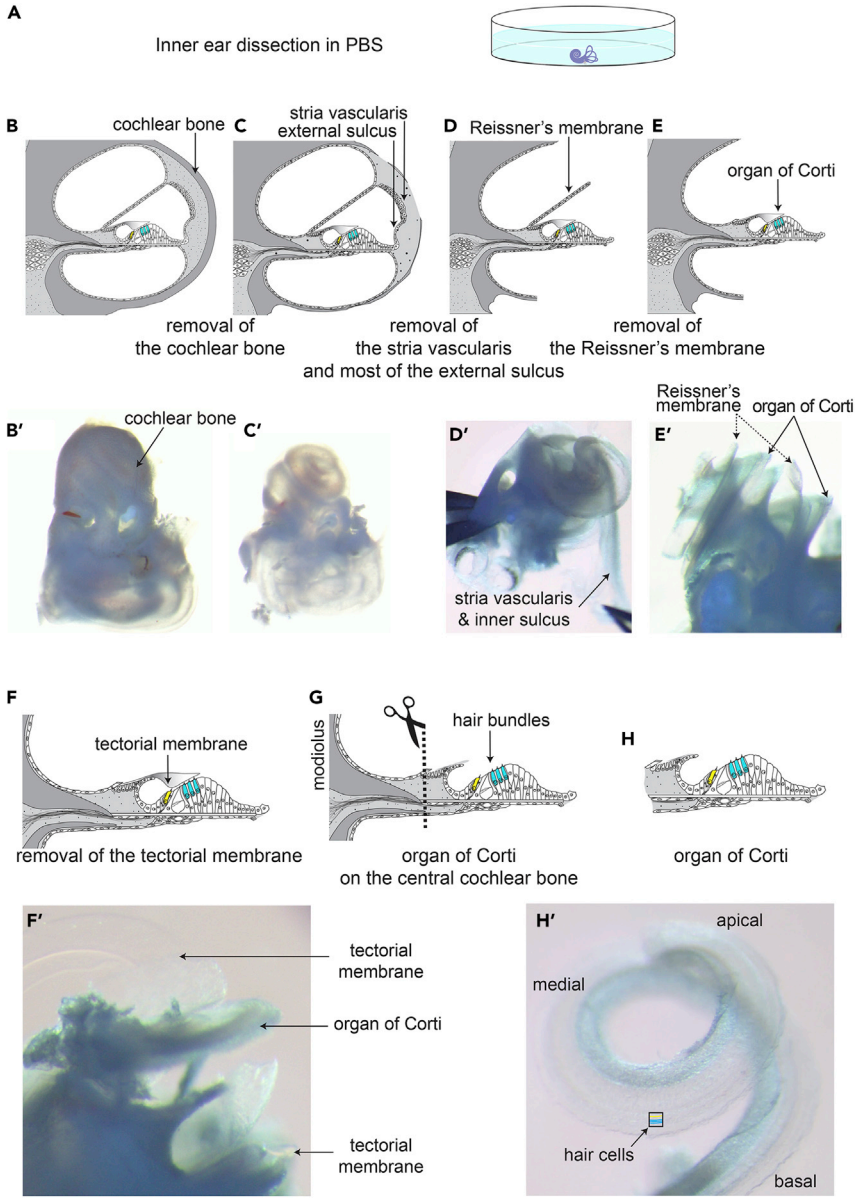

Figure 3. Fine dissection of the inner ear

(A) The inner ear (in this example, P6) is placed in a fresh plate containing PBS.

(B and B′) The cochlear bone is chipped away, starting from the apex of the cochlea and working toward the base.

(C, C′, and D′) The stria vascularis is peeled away from the organ of Corti, starting from the apex.

(D, E, and E′) Reissner's membrane, which sits above the organ of Corti, is removed.

(F and F′) The tectorial membrane is removed, allowing direct access to the hair bundles at the apical surface of the hair cells.

(G, H, and H′) (G) The organ of Corti is separated from the central cochlear bone, known as the modiolus, resulting in (H and H′) the fully dissected organ of Corti. Graphics of the inner ear were adapted with permission from Shutterstock.

17. Remove Reissner's membrane (a thin, flat membrane that juts out above the organ of Corti) using #55 forceps.

Note: Reissner's membrane must often be removed in multiple steps along the cochlea (Figures 3D and 3E).

18. Remove the tectorial membrane (a nearly invisible membrane that sits closely on top of the hair cells) using #55 forceps (Figures 3F and 3F′).

Note: The tectorial membrane is translucent and often very difficult to see. For mice at P11 or older, it can be seen when looking at the organ of Corti from the side. For younger mice, the tectorial membrane might come off in pieces and might be difficult to see unless it is actively being removed. In either case, it is best to gently grab the tectorial membrane using fine (#55) forceps at the location where it is expected to be (above the hair cells). Once the membrane has been grabbed, pull it away from the tissue and it should become visible as it unrolls. By P11, it should be possible to remove nearly the whole tectorial membrane in one attempt.

19. Using forceps, remove the organ of Corti from the central bone spire known as the modiolus by gently puncturing or tearing the tissue between the organ and the bone.

Note: The organ of Corti should easily be released from the modiolus when dissecting. (Figures 3G, 3H, and 3H′). Alternatively, the organ can be left on the modiolus during staining for ease of handling.

Step 3: Immunostaining and mounting

Timing: 24 h

The steps for hair bundle immunostaining include permeabilizing (opening pores in the membrane), blocking (preventing nonspecific antibody binding), primary antibody incubation (targeting the protein of interest), secondary antibody incubation (providing immunofluorescence to the target of interest), phalloidin incubation (staining the actin filaments of the stereociliary cytoskeleton), and mounting the sample for ease of imaging.

Note: Opening pores in the stereociliary membrane via permeabilization allows antibodies to access intracellular proteins. The tissue must be blocked to prevent nonspecific binding of antibodies. A low amount of mild detergent (Tween-20) is maintained in all solutions to reduce the surface tension on the sample which prevents it from adhering to surfaces.

Note: Here, we stained the protein espin-1 with a commercially available antibody (Clone 31, BD Biosciences), which generates consistently specific and strong labeling in the hair bundle. Espin-1 is an actin-bundling protein located at the tips of the stereocilia. Although espin-1 staining varies during development, it is expressed at the stereocilia tips as early as P0 (Salles et al., 2009) (Figure 4A).

20. Using a small spoon, transfer the organ of Corti to a glass Micro-Test Staining Dish containing 200 μL permeabilization buffer (4% BSA in PBS with 0.05% Triton X-100) (Figure 4B).

Note: The glass dishes described in this protocol are used because they do not strongly bind with antibodies and can be reused. Some plastics like polypropylene adsorb proteins including antibodies and could potentially decrease staining efficiency. However, if disposable plastic must be used, choose a plastic treated for low binding. In terms of tube shape, we suggest 2-mL tubes with pointed bottoms which allows easy visualization of the samples and requires smaller amounts of diluted antibody.

Note: All reused materials, such as glass dishes, spoons, dissecting equipment, etc., are cleaned after each use with a thorough rinse with Milli-Q water.

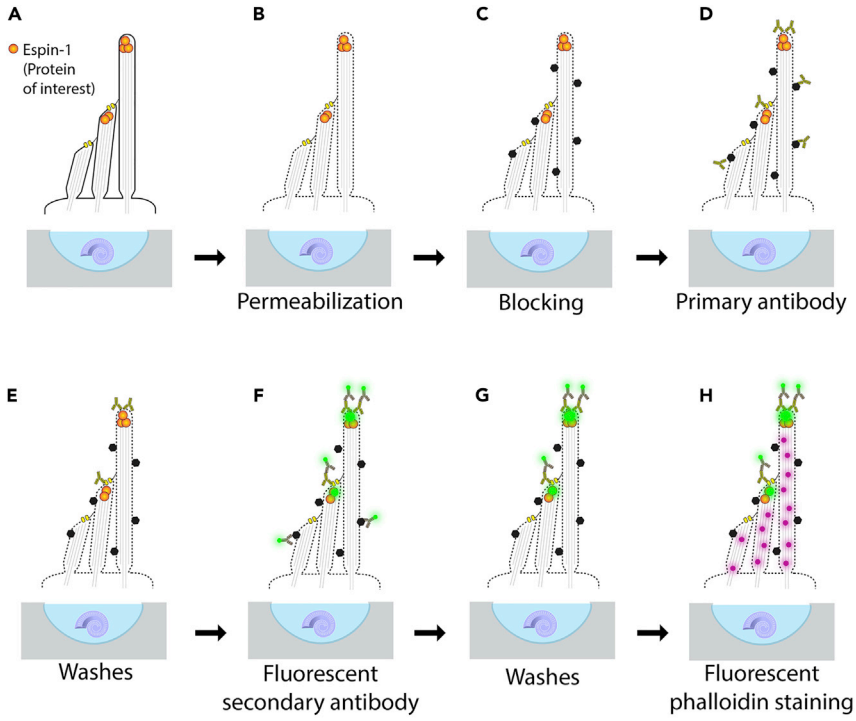

Figure 4. Immunostaining the organ of Corti

(A) To identify a protein of interest (in this case, espin-1), the tissue is first placed in a Micro-Test Staining Dish well containing PBS.

(B) The PBS is removed, keeping a small drop to cover the tissue, and permeabilization buffer is added for 20 min at 25°C, shaking.

(C) Permeabilization buffer is removed, and blocking buffer is added for 1 h at 25°C, shaking.

(D) After discarding the blocking buffer, the tissue is incubated for 12–16 h at 4°C, shaking with buffer containing diluted primary antibodies.

(E) The primary antibody solution is removed, and the tissue is washed with blocking buffer three times 5 min at 25°C, shaking.

(F) After the last wash, buffer containing diluted fluorescent secondary antibodies is added for 1 h at 25°C, shaking.

(G) Secondary antibody is discarded, and the tissue is washed with blocking buffer once for 5 min at 25°C, shaking.

(H) Blocking buffer is removed, and buffer containing diluted fluorescently labeled phalloidin (to stain the actin core) is added for 20 min at 25°C, shaking.

21. Incubate in permeabilization buffer for 20 min at 25°C with gentle shaking on an orbital shaker (60 rpm for this and all following shaking steps).
22. Remove permeabilization buffer, leaving a small drop of buffer covering the sample.
23. Add 200 μL blocking buffer (4% BSA in PBS with 0.05% Tween-20) for 1 h at 25°C with gentle shaking (Figure 4C).

Pause point: Blocking can be performed for longer periods, even for 12–36 h at 4°C. However, as different incubation times can affect the intensity of staining and/or the background, always use the same protocol to compare samples of different genotypes.

24. Remove the blocking buffer, leaving a small drop of solution covering the sample.
25. Add 200 μL of dilution buffer (1% BSA in PBS with 0.05% Tween-20) containing the diluted primary antibody (Figure 4D).

Note: The espin-1 staining in this protocol was performed at a 1:200 dilution. Multiple antibody dilutions should be tested to find the ideal condition when initializing a protocol (See

troubleshooting section). Start with the dilution recommended by the manufacturer of your antibody of interest and test 2–3 times sequential higher dilutions.

Note: Antibody staining of the protein of interest and phalloidin staining of the actin core of the stereocilia allow clear localization of hair bundle proteins.

26. Incubate at 4°C for 12–16 h with gentle shaking.

Note: Place the sample plate in a Western blot box or empty pipette tip box with a lid to prevent liquid evaporation during longer incubations. To saturate the air in the box with water and further limit evaporation, a Kimwipe dampened with distilled water can be placed in the bottom of the box below the Micro-Test Staining Dish. Finally, the box can be sealed with parafilm.

27. Remove the primary antibody solution, leaving a small drop of solution covering the sample.
28. Wash 3 × 5 min with 200 μL dilution buffer at 25°C with gentle shaking (Figure 4E).
29. Remove the dilution buffer, leaving a small drop of solution covering the sample.
30. Add 200 μL of dilution buffer (1% BSA in PBS with 0.05% Tween-20) containing the diluted secondary antibody (Figure 4F).

Note: For the staining performed in this protocol, a 1:500 dilution of secondary antibody was used.

31. Incubate at 25°C for 1 h, protected from light, with gentle shaking.

Note: From this point on, the samples should be protected from light as much as possible to reduce photobleaching of the fluorophores. This can be done by placing the sample plate in a Western blot box and covering the samples with aluminum foil during the incubation steps until mounting.

32. Wash 1 × 5 min with dilution buffer at 25°C with gentle shaking (Figure 4G).
33. Remove the dilution buffer, leaving a small drop of solution covering the sample.
34. Add 200 μL of dilution buffer (1% BSA in PBS with 0.05% Tween-20) with diluted phalloidin (1:500 dilution of 6.6 μM solution, methanol reconstituted, or 1:1000 dilution of 66 μM solution, DMSO reconstituted) (Figure 4H).

Note: DMSO reconstitution of phalloidin may be more stable for long-term storage. We have found excellent results with both solvents.

35. Incubate at 25°C for 20 min, protected from light, with gentle shaking.

Note: The intensity of phalloidin staining can be increased by extending the incubation time; however, this might result in oversaturated staining of the stereociliary actin core (See troubleshooting section).

36. Wash 5 × 5 min with dilution buffer at 25°C with gentle shaking.

(Methods video S5).

37. Remove the organ of Corti from the modiolus if still attached.
38. Perform final trims on the tissue including removing any remaining pieces of Reissner's membrane.
39. Cut the tissue into "turns", or curved portions of the organ that can lie flat on the slide without overlapping with themselves (Figure 5A).

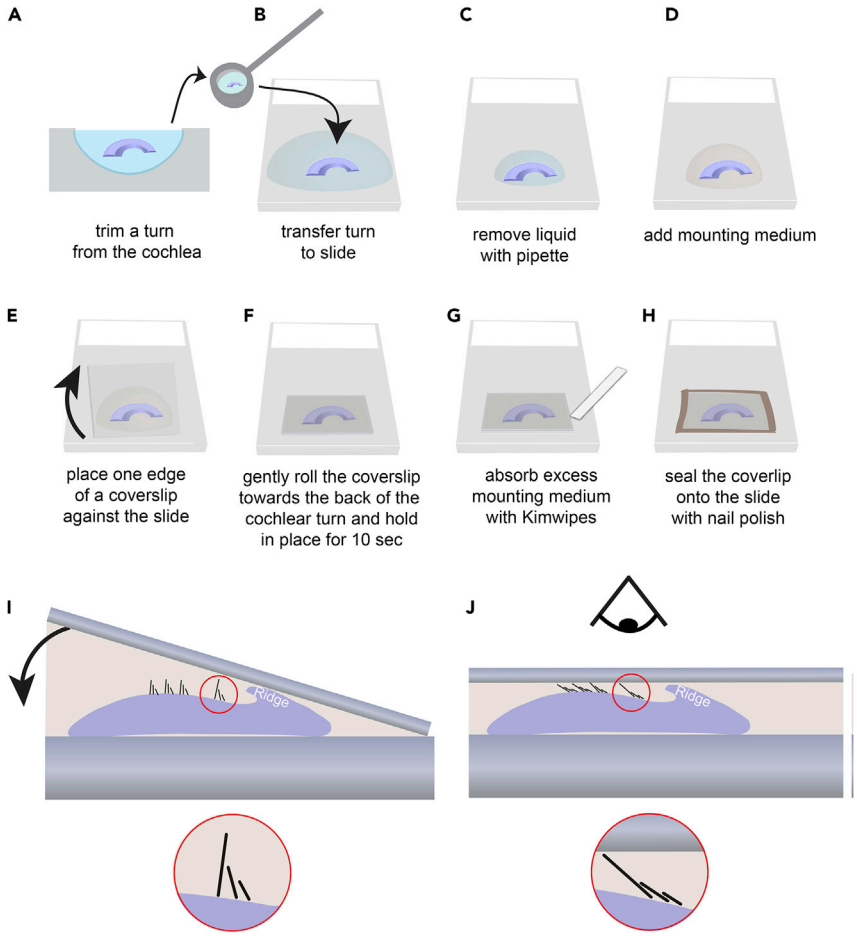

Figure 5. Whole tissue mounting of the organ of Corti

(A) Perform final trims on the tissue, including cutting it into turns that will not overlap with themselves when mounted flat.
(B) Using a small spoon, transfer the turn to the slide.
(C) Remove excess liquid with a pipette, leaving a small drop of liquid covering the tissue.
(D) Add a drop of mounting medium on top of the tissue and orient the tissue with the hair bundles facing upward.
(E) Place one edge of the coverslip against the slide.
(F) In the direction of sound-induced force deflection for a large region of hair bundles, gently roll the coverslip onto the turn, and hold firmly in place for 10 s.
(G) Absorb any excess mounting medium with a Kimwipe.
(H) Seal the coverslip in place with fingernail polish to prevent movement while the mounting medium is hardening.
(I and J) Close-up visualization of how rolling the coverslip in the direction of hair bundle deflection can cause hair bundles to lie flat in an optimal orientation to visualize all stereocilia. Note the ridge located on the surface of the epithelium can be used as a landmark to verify sample orientation.

Note: By properly trimming and mounting the whole organ of Corti, the hair bundle and individual stereocilia can be visualized.

Note: For the full organ of Corti, the tissue can be separated into three turns: an apical turn, a middle turn, and a basal turn.

40. Using the smallest spoon possible, transfer the turns to the slide (Figure 5B).

Note: As an alternative to using a spoon, a glass Pasteur pipette with the tip enlarged using a glass etcher could also be used. However, plastic transfer pipettes should be avoided, if possible, as the tissue can easily stick to the sides of the pipette.

41. Remove excess liquid with a pipette but keep a small drop to cover the tissue (Figure 5C).
42. Add a drop of mounting medium (Prolong Gold or similar is ideal) onto the turn (Figure 5D).

⚠ CRITICAL: Make sure to remove as much of the dilution buffer as possible as it can alter the refraction index of the mounting medium.

CRITICAL: Make sure the turn does not come in contact with air as this might potentially affect the hair bundle's integrity.

Note: Alternatively, the mounting medium can be added to the slide first, and the turn can be placed in the medium using the smallest spoon possible. This can only be done if the quantity of liquid transferred with the turn is extremely limited.

43. Under the stereomicroscope, use forceps to orient the sample facing upward and push it to the bottom of the mounting medium droplet until it sits on the glass slide.

⚠ CRITICAL: Make sure the turn is facing upward when mounting. When the turn is observed from the side, there should be a central ridge running through the organ of Corti on the same side as the hair bundles (Figures 1E and 5I). This ridge should be facing upward to image hair bundles (for a demonstration of steps 39–47, see Methods video S5).

44. Selecting a large region of hair bundles, slowly roll the coverslip down onto the sample in the direction that the hair bundles would be deflected by sound-induced forces (from the intrados of the arched tissue to the extrados) (Figures 5E, 5F, 5I, and 5J).

⚠ CRITICAL: Perform this step slowly. If the coverslip is rolled too quickly, it can move the sample, which may end up damaging it.

45. Using two hands, firmly hold the coverslip in place for 10 s to ensure that the bundles are lying flat.

Note: Holding the coverslip firmly in place helps to bring the hair bundles very close to the glass coverslip. Not only does this help to push the hair bundles to lie flat, allowing imaging of the full hair bundle, but it reduces the distance between the hair bundle and the coverslip, improving the imaging resolution.

46. Use a Kimwipe to remove any excess mounting medium from the edges of the coverslip (Figure 5G).
47. If appropriate, seal the coverslip with nail polish.

Note: If imaging immediately or if using a non-hardening mounting medium, the coverslip should be sealed with nail polish. Regardless, the coverslip can be sealed at the corners to prevent movement before allowing the mounting medium to harden at 4°C (Figure 5H).

Note: With this mounting technique, many of the hair bundles will be very close to the coverslip with little mounting medium between the sample and the glass. Because of this, even when using a hardening mounting medium, excellent images can be obtained immediately after mounting if the coverslip is sealed into place, since the refractive index of the mounting medium will not have a major impact on the resolution of those specific bundles. However, for bundles that are not lying directly against the coverslip, better images can be obtained after allowing the mounting medium to cure (e.g., for Prolong Gold, 24 h) before sealing.

Pause point: After mounting, the samples can be stored in the dark at 4°C until imaging. Imaging should be performed as soon as possible for best results but can be performed up

to at least a week later. Although excellent images might be obtained at later time points, oxidation or photobleaching of fluorophores might potentially affect the outcomes.

Step 4: Imaging the hair bundle

Timing: about 3 h

Immunofluorescence imaging of a properly mounted tissue can provide clear insight into the localization of a protein of interest (Figure 6).

48. At the eye piece, locate and bring the turn into focus using the phalloidin emission channel under 10× magnification.
49. Still at the eye piece, switch to at least a 63× objective.

Note: Make sure to add the proper immersion medium for the objective (e.g., oil, glycerol).

Note: The numerical aperture (NA) of the lens being used will directly affect the attainable lateral resolution. The larger the numerical aperture, the greater the maximum resolution that can be obtained. In our protocol, our 10× objective has an NA of 0.45 and our 63× objective has an NA of 1.4.

50. Visually scan the sample and identify regions with hair bundles properly oriented for imaging (lying flat with the inner stereociliary rows on top).
51. Once the region of interest is centered, switch to acquisition mode.
52. Quickly adjust the laser intensity and gain in the phalloidin channel such that no pixels are oversaturated.

Note: Lower laser intensity causes less bleaching and is less likely to cause bleed-through into other channels. For strong staining (such as phalloidin), laser power can often be below 0.5%. For less intense staining, it is still best to attempt to keep the laser power below 10% if possible. The maximum laser power used was approximately 5.5 μW for the 488 laser and 49.8 μW for the 561 laser.

53. Quickly adjust the laser intensity and gain in the channel pertaining to the protein of interest such that no pixels are oversaturated.
54. Select the z-stack region and step size and crop the image to the bundles of interest.

Note: Minimum z step size should be checked with your imaging system. Different microscopes have different limitations. The z stack region should encompass the full hair bundle. For reference, we collected data for this image with a z depth of 2.24 microns with a step depth of 330 nanometers. However, the z depth will be different from bundle to bundle, depending on its size and how flat it is lying.

55. Potentially adjust parameters such as the pinhole (a larger pinhole increases the intensity and signal-to-noise ratio, but decreases the lateral resolution), averaging (increases the signal-to-noise ratio), accumulation (increases signal) and the speed of acquisition to obtain optimal images.
56. Acquire the image z-stack.

Note: After imaging, adjusting the image parameters post-imaging can help to reveal cleaner signal and reduce background.

57. After imaging, adjust the high and low intensity levels using an image processing software (e.g., Imaris or Fiji) to obtain low background and clear signal.

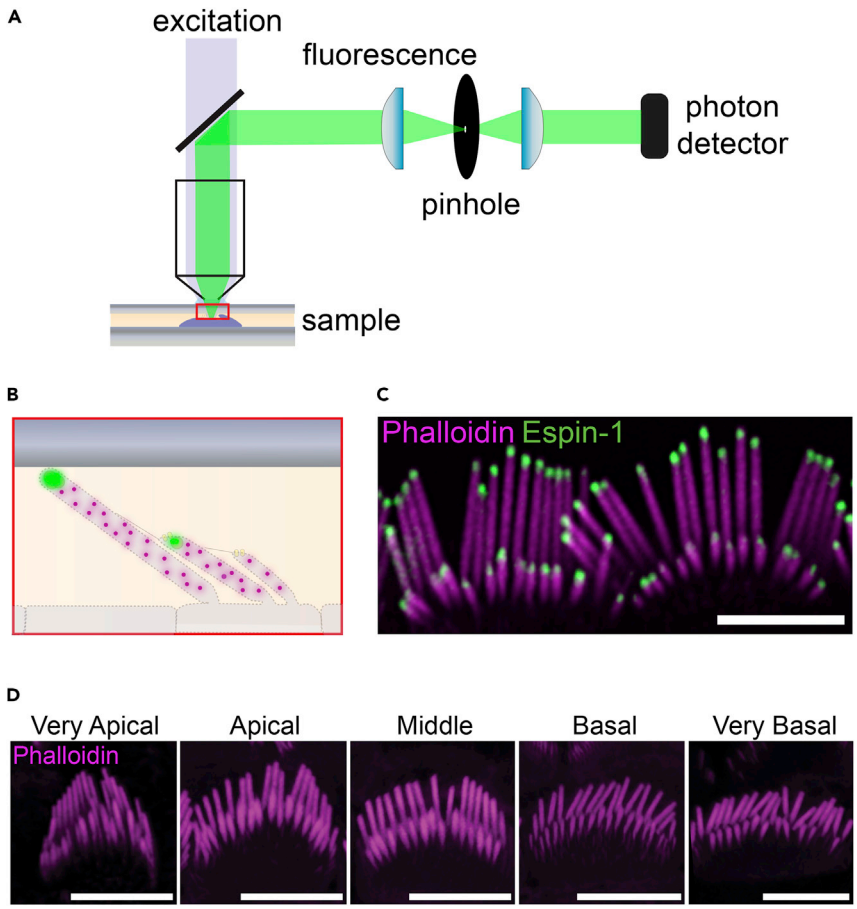

Figure 6. Immunofluorescence imaging of the bundles

(A and B) (A) A confocal microscope (or better) is used to localize (B) fluorescently labeled proteins of interest located within the stereocilia in samples mounted below a coverslip. Note that the stereocilia are close to the apical surface of other cells.

(C) Example of espin-1 immunostaining at stereociliary tips in P11 mouse inner hair cell hair bundles.

(D) Example images of phalliodin-labeled inner hair cell hair bundles from different regions (very apical, apical, middle, basal, and very basal) throughout a P7 organ of Corti.

Scale bars for (C) and (D): 5 mm.

58. Either select a z-slice containing the staining of interest, or select a range of z-slices to generate a maximum intensity projection, or generate a 3D-reconstruction of the image (for an example of 3D-reconstructed bundles, see Miller et al., 2021) to localize the protein of interest.

EXPECTED OUTCOMES

Using this protocol, it should be possible to obtain consistent and reproducible results with a given antibody. Hair bundles should retain their structure and lie flat for imaging. Here we show an example of immunofluorescence localization of espin-1 in inner hair cell hair bundles at P11 (Figures 6B and 6C).

LIMITATIONS

After P8, the mouse cochlear bone starts to harden, which makes the dissection challenging and obtaining the full organ of Corti difficult. For adult cochleae, the bone is so hard that it is only possible to remove the apical turn. To obtain the full organ, the adult cochlea must be decalcified with EDTA (Montgomery and Cox, 2016); however, treatment with calcium chelators can affect antigenicity.

Obtaining specific regions where the bundles are lying flat is an additional limitation. For mice younger than P8, it is easy to find large regions of the cochlear turns with hair bundles lying flat. Even at slightly older ages, usually only certain stretches of hair bundles will lie flat in a given sample. It can potentially be misleading to compare expression patterns if not using precisely the same region within the tonotopic gradient.

While minor damage to some hair bundles could occur from the preparation, the majority of hair bundles all along the cochlear apico-basal axis will be preserved using this protocol.

TROUBLESHOOTING

Problem 1

Hair bundles are splayed or incorrectly oriented (step 50).

Potential solution

The sample might have been in contact with air or the mounting step might have been performed incorrectly. Take care that the sample is always immersed in liquid and that the mounting steps are carefully followed.

Problem 2

The phalloidin staining is low or inconsistent (step 50).

Potential solution

Increase the percentage of the permeabilization detergent. Increase the phalloidin concentration, extend the incubation time for the sample in phalloidin, or replace the current phalloidin stock. Make sure that the tissue is fully immersed in all solutions, rather than floating on top.

Problem 3

Images contain high background staining (steps 53 and 55).

Potential solution

For each new staining condition, multiple concentrations for the primary and secondary antibodies should be tested. Similarly, a different blocking solution or a longer blocking time might reduce background. The number and length of washes could also be increased to decrease background.

Problem 4

No visible hair bundles in the sample (steps 48 and 50).

Potential solution

Sample might be mounted upside down and typically cannot be used. In the future, make sure the cochlear turn is facing upward. This can be verified by looking at the turn from the side while mounting. A small ridge runs along the organ of Corti on the side of the tissue containing the hair bundles, and this ridge should face upward when mounted (see Methods video S5 for an example).

Problem 5

Bleed-through between imaging channels (steps 55 and 57).

Potential solution

Reduce the laser power and adjust the cutoff points for the detectors to make sure they do not overlap. Use different fluorophores with emission spectra sufficiently distant from each other.

Problem 6

No fluorescence visible (steps 53 and 55).

Potential solution

Adjust the antibody concentrations and permeabilization conditions. Check positive control to make sure the conditions are effective. If using endogenous GFP or similar fluorescent tags, note that fixation conditions can affect their ability to fluoresce.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be provided by the lead contact, Nicolas Grillet (ngrillet@stanford.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101431.

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AUTHOR CONTRIBUTIONS

N.G. and K.K.M. formulated the project. K.K.M. and P.W. carried out methodology and investigation. N.G. and K.K.M. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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