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cover letter

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To: Dr. Ron Myers, Ph.D.
Editor in Chief
JOVE

May 21, 2019

Dear Dr. Myers,

I am very glad to accept your invitation for submission our manuscript entitled *"*Cochlear surface preparations in the adult mouse*"* by Qiao-Jun Fang, Renjie Chai, and Su-Hua Sha for publication in *JOVE.*

Surface preparations of cochlear epithelia, in combination with immunolabeling techniques and confocal imagery, are a very useful tool for the investigation of cochlear pathologies, including losses of ribbon synapses and sensory hair cells, changes in protein levels in sensory hair cells and supporting cells, and hair cell regeneration. With the availability of molecular and genetic information and the ability to manipulate genes by knockout and knock-in techniques, mice have been widely used in biological research, including in hearing science. However, the adult mouse cochlea is tiny and the cochlear epithelium is encapsulated in a bony labyrinth, making micro-dissection difficult. Although dissection techniques have been developed and used in many laboratories, in this manuscript we describe our modified micro-dissection method, which we consider easier and more convenient.

The work described in this manuscript is original and has not been published, nor is it submitted to any other journal for publication. I am sincerely appreciative for your invitation.

Sincerely,

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TITLE:

Cochlear Surface Preparation in the Adult Mouse

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KEYWORDS:

cochlear surface preparation, whole mount dissection, sensory hair cells, cochlear ribbon synapses, adult mice, immunolabeling, immunohistochemistry, fluorescent staining

SUMMARY:

This article presents a modified cochlear surface preparation method that requires decalcification and use of a cell and tissue adhesive to adhere the pieces of cochlear epithelia to 10 mm round cover slips for immunohistochemistry in adult mouse cochleae.

ABSTRACT:

Auditory processing in the cochlea depends on the integrity of the mechanosensory hair cells. Over a lifetime, hearing loss can be acquired from numerous etiologies such as exposure to excessive noise, the use of ototoxic medications, bacterial or viral ear infections, head injuries, and the aging process. Loss of sensory hair cells is a common pathological feature of the varieties of acquired hearing loss. Additionally, the inner hair cell synapse can be damaged by mild insults. Therefore, surface preparations of cochlear epithelium, in combination with immunolabeling techniques and confocal imagery, are a very useful tool for the investigation of cochlear pathologies, including losses of ribbon synapses and sensory hair cells, changes in protein levels in hair cells and supporting cells, hair cell regeneration, and determination of report gene expression (i.e., GFP) for verification of successful transduction and identification of transduced cell types. The cochlea, a bony spiral-shaped structure in the inner ear, holds the auditory sensory end organ, the organ of Corti (OC). Sensory hair cells and surrounding supporting cells in the OC are contained in the cochlear duct and rest on the basilar membrane, organized in a tonotopic fashion with high-frequency detection occurring in the base and low-frequency in the apex. With the availability of molecular and genetic information and the ability to manipulate genes by

knockout and knock-in techniques, mice have been widely used in biological research, including in hearing science. However, the adult mouse cochlea is miniscule, and the cochlear epithelium is encapsulated in a bony labyrinth, making microdissection difficult. Although dissection techniques have been developed and used in many laboratories, this modified microdissection method using cell and tissue adhesive is easier and more convenient. It can be used in all types of adult mouse cochleae following decalcification.

INTRODUCTION:

The cochlea is dedicated to the detection of sound and responsible for hearing. The cochlear duct is coiled in a spiral shape in the bony labyrinth and holds the auditory sensory end organ, the organ of Corti (OC). The OC rests on the basilar membrane, making up the cochlear epithelium, with a length of about 5.7 mm when uncoiled in adult CBA/CaJ mice[1,2]. Because the OC is tonotopically organized with high frequencies detected in the base and low frequencies in the apex, the cochlear epithelium is often divided into three parts for analytical comparisons: the apical, middle, and basal turns corresponding to low, middle, and high frequency detection, respectively. In addition to an array of supporting cells, the OC is composed of one row of inner hair cells (IHCs) located medially and three rows of outer hair cells (OHCs) located laterally with respect to the cochlear spiral.

Correct auditory processing depends on the integrity of the sensory hair cells in the cochlea. Damage to or loss of sensory hair cells is a common pathological feature of acquired hearing loss, caused by numerous etiologies such as exposure to excessive noise, the use of ototoxic medications, bacterial or viral ear infections, head injuries, and the aging process[3]. Additionally, the integrity and function of the inner hair cell/auditory nerve synapses can be impaired by mild insults[4]. With the availability of molecular and genetic information and manipulation of genes by knockout and knock-in techniques, mice have been widely used in hearing science. Although the adult mouse cochlea is minuscule and the cochlear epithelium is surrounded by a bony capsule resulting in technically difficult microdissections, surface preparations of the epithelium in combination with immunolabeling or immunohistochemistry and confocal imagery have been broadly used for investigation of cochlear pathologies, including losses of ribbon synapses and hair cells, changes in levels of proteins in sensory hair cells and supporting cells, and hair cell regeneration. Cochlear surface preparations have also been used to determine the pattern of expression of reporter genes (i.e., GFP) and confirm successful transduction and identify transduced cell types. These techniques have been previously used for the study of molecular mechanisms underlying noise-induced hearing loss using adult CBA/J mice[5–9].

Unlike immunohistochemistry using paraffin sections or cryosections to obtain small cross-sectional portions of the cochlea that contain three outer hair cells (OHCs) and one inner hair cell (IHC) on each section, cochlear surface preparations allow visualization of the entire length of the OC for counting sensory hair cells and ribbon synapses and immunolabeling of sensory hair cells corresponding to specific functional frequencies. **Table 1** shows the mapping of hearing frequencies as a function of distance along the length of the cochlear spiral in adult CBA/J mouse according to studies from Muller[1] and Viberg and Canlon[1,2]. Cochlear surface preparations have been widely used for investigation of cochlear pathologies[4–15]. The whole-mount cochlear

dissection method was originally described in a book edited by Hans Engstrom in 1966[16]. This technique was subsequently refined and adapted to a variety of species as described in the literature by a number of scientists in hearing science[10–13,15,17] and by the Eaton-Peabody Laboratories at Massachusetts Eye and Ear[18]. Recently, another cochlear dissection method was reported by Montgomery et al.[19]. Microdissection of the cochlea is an essential and critical step for cochlear surface preparations. However, dissecting mouse cochleae is a technical challenge and requires considerable practice. Here, a modified cochlear surface preparation method is presented for use in adult mouse cochleae. This method requires decalcification and use of cell and tissue adhesive (i.e., Cell-Tak) to adhere the pieces of cochlear epithelium to 10 mm round cover slips for immunolabeling. Cell and tissue adhesive has been widely used for immunohistochemistry[20]. This modified cochlear microdissection method is relatively simple compared to those previously reported[18,19].

PROTOCOL:

All research protocols involving male adult CBA/J mice at the ages of 10–12 weeks and C57BL/6J mice at the ages of 6–8 weeks were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina (MUSC). Animal care was under the supervision of the Division of Laboratory Animal Resources at MUSC.

NOTE: For the procedures presented below, mice are anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injections. Mice are decapitated after the animal no longer responds to painful stimuli, such as a toe pinch.

1. Extraction of temporal bones

1.1. Decapitate the mouse immediately postmortem with surgical scissors (17 cm long) and cut the skull bone with scissors from the posterior aspect forward along the center line of the skull after exposing the skull bone by pulling the skin anteriorly.

1.2. Remove the brain tissue using forceps and manually remove the temporal bones with the thumb and index finger for mice three months of age or older. Use small surgical scissors (11 cm long) to cut the temporal bone of mice younger than three months old.

1.3. Put the temporal bone into a Petri dish (30 mm in diameter) containing ice-cold fresh 4% paraformaldehyde (PFA) solution dissolved in phosphate-buffered saline (PBS), pH 7.4.

NOTE: Prepare fresh 4% PFA solution and adjust the pH to 7.4 just before fixing the temporal bones, because improper pH balancing of the PFA solution will decrease the quality of the immunolabeling.

2. Fixation and perfusion

2.1. Remove the stapes from the oval window and puncture the round window membrane with size #5 forceps. Under a stereo dissection microscope make a small hole into the apex of the cochlea using a 27 G needle connected to a 1 mL syringe.

2.2. Gently and slowly perfuse the cochlea with 4% PFA solution via the round and oval windows until the solution washes out of the small hole at the apex. Transfer the cochlea (one or two cochleae per vial) to 20 mL volume scintillation vials containing 10 mL of 4% PFA solution.

2.3. Gently agitate the scintillation vials at room temperature (RT) for 2 h and leave overnight in a refrigerator (4 °C) on a rotator.

NOTE: The time of fixation can be adjusted depending on the antibody used. For example, limiting the fixation to 1.5 h will allow for successful immunolabeling of post synaptic terminals when using the GluA 2 antibody.

3. Decalcification of the temporal bone

3.1. Remove the PFA solution and wash the cochleae with fresh PBS 3x for 5 min each.

3.2. Add 20 mL of 4% ethylenediaminetetraacetic acid disodium salt (EDTA) solution (pH 7.4) to the scintillation vials and keep in a refrigerator for 48–72 h on a rotator with gentle agitation. Change the EDTA solution daily until the cochleae are decalcified. Check if the cochleae are decalcified by touching the bony vestibular portion with forceps to assess for elasticity or simply cut a small piece from the edge of the vestibular portion. If such cut results in a crushed piece, the cochlea is not decalcified.

NOTE: The EDTA solution may interfere with the immunoreaction of primary antibodies depending on the solution concentration. Prepare 4% EDTA in PBS and adjust the pH to 7.4. For consistent decalcification of temporal bones, fresh EDTA solution is used.

3.3. Change the solution to PBS for microdissection after decalcification is complete.

NOTE: If the cochleae are not sufficiently decalcified, dissection of the cochleae cannot be performed.

4. Microdissection of the cochlear epithelium

NOTE: Once decalcification is complete, microdissection of cochlear epithelium for immunolabeling needs to be performed as soon as possible. In a clean 30 mm Petri dish containing PBS, the inner ear is oriented in the following manner: in reference to Petri dish's top (lid) and bottom, the cochlear round and oval windows face the top. In reference to dissector, the cochlear portion is oriented toward the front (away from dissector) and vestibular portion toward the back (near dissector). The following describes the steps in detail.

4.1. Hold the vestibular portion of the temporal bone with forceps and cut the apical turn with the scalpel at a 45° angle as indicated by the red line (**Figure 1a**).

4.2. Cut vertically along the faint line between the round window and the oval window, as indicated by the red line (**Figure 1b**) to separate the cochlea from the vestibular portion (**Figure 1c**).

NOTE: This cochlear portion contains the middle, basal, and hook portions of the epithelium.

4.3. Place the cochlear portion with the basal turn toward the bottom and the middle turn toward the top of the Petri dish and cut the bony capsule and lateral wall of the middle turn toward the end from which the apical section was removed, as indicated by the red line (**Figure 1d,e**).

4.4. Continue cutting to completely separate the middle portion from the basal and hook regions (**Figure 1f,g**).

NOTE: The hook region of the cochlea is the end of the cochlear epithelium corresponding to sensitivity to tones 48 kHz and higher in mice.

4.5. Put the basal and hook portion with the basilar membrane site oriented toward the bottom of the dish and vertically cut the modiolus off the hook region to remove the modiolus as indicated by the vertical red line (**Figure 1g**).

NOTE: The modiolus is the conical-shaped central axis of the cochlea that consists of spongy bone and cochlear nerve as well as the spiral ganglion. It may be preferable to perform this cut with scissors.

4.6. Cut the basal and hook regions as the other red line indicates in **Figure 1g** to separate the hook portion (**Figure 1h**).

NOTE: The cochlea has now been separated into apical, middle, basal, and hook portions. The next steps will be the final dissection of each of the individual turns, using the middle turn as an example.

4.7. Cut away the relatively large portions of bony capsule and lateral wall tissue of the middle turn as indicated by the red line (**Figure 1i**).

NOTE: The lateral wall of the cochlear duct is formed by the spiral ligament and the stria vascularis.

4.8. Hold the lateral wall with forceps to align the bony capsule and lateral wall with the bottom of the Petri dish and cut these tissues from the basilar membrane side. Then flatten the specimen

to orient the sensory hair cell surface site up. Trim away the rest of the bony capsule and lateral wall (**Figure 1j**).

4.9. Remove the tectorial membrane using forceps to completely separate the middle region (**Figure 1k**).

4.10. Repeat steps 4.7–4.9 for the final dissections of the remaining turns or regions as shown in **Figure 1l**.

4.11. Prepare a 10 mm round coverslip for adhesion of the sensory epithelium. Use a pipette to hand-spread 0.5 µL of cell and tissue adhesive on the center of the round coverslip. After drying (1–2 min at RT), put the coverslips in PBS in Petri dishes.

4.12. Stick all four pieces of the sensory epithelium on the 10 mm round coverslip in the Petri dish. Then hold one edge of the coverslip to transfer it to a four-well dish for immunolabeling or immunohistochemistry (**Figure 1m**).

NOTE: **Figure 2** illustrates the location of the major cuts.

5. Immunolabeling for cochlear synapses

NOTE: The protocol for immunolabeling for cochlear synapses followed in this study has been previously described[13]. Presynaptic ribbons were labeled with a CtBP2 antibody (mouse anti-carboxyl-terminal binding protein 2 IgG1, labeling the B domain of the RIBEYE scaffolding protein). Post synaptic terminals were labeled with GluA2 antibody (mouse anti-glutamate receptor 2 IgG2a, labeling subunits of the AMPA receptor).

5.1. Wash the sensory epithelium with PBS 3x for 5 min each wash in a four-well dish and then add 2 mL of 2% nonionic surfactant (i.e., Triton-X 100) into the dish for 30 min at RT on a rotator.

5.2. Remove the nonionic surfactant solution from the dish using a pipette and add 100 µL of blocking solution containing 10% normal goat serum to each well for 1 h on a rotator with gentle agitation at RT.

5.3. Remove the blocking solution from each well, wash with PBS 3x for 5 min each wash under gentle agitation at RT.

5.4. Add 100 µL of the primary antibodies diluted with PBS to each well: mouse anti-GluA2 IgG2a (1:2,000), mouse anti-CtBP2 IgG1 (1:400). Cover each four-well dish with its lid and place into a large humidified container that protects from light. Incubate at 37 °C for 24 h under gentle agitation.

NOTE: As an option, rabbit anti-myosin VIIa (1:400) for immunolabeling sensory hair cells can be added.

5.5. Wash 3x with PBS for 5 min each wash with gentle agitation at RT.

5.6. Add 100 µL of the secondary antibodies Alexa 594 goat anti-mouse IgG1a (1:1,000), Alexa 488 goat anti-mouse IgG2a (1:1,000) diluted with PBS to each well. Cover each four-well dish with its lid and place into a large humidified container that protects from light. Incubate at 37 °C for 2 h.

NOTE: If myosin VIIa is used, add Alexa 350 goat anti-rabbit (1:200).

5.7. Wash 3x with PBS for 5 min. Then, transfer the 10 mm round coverslip onto a slide with the samples on top.

5.8. Carefully add 8 µL of Fluoro-Gel with Tris buffer into the center of the coverslip. Then hold the edge of another 10 mm round coverslip with forceps to mount on top, sandwiching the two coverslips together.

5.9. Use nail polish to seal the slides, put them in a cardboard slide folder, and store in the refrigerator.

NOTE: If some Fluoro-Gel leaks out between the coverslips during mounting, clean the edges of the coverslips before sealing the slide with nail polish. Confocal images need to be taken within 7 days.

REPRESENTATIVE RESULTS:

Surface preparations of the cochlear epithelium, in combination with immunolabeling and confocal imaging, have been used broadly in hearing science for the investigation of cochlear pathologies, such as quantification of ribbon synapses, sensory hair cells, and protein expression in sensory hair cells[5–8]. Although the dissection of adult mouse cochleae for surface preparations is not simple, new graduate students are able to learn this modified method after practicing with 10–15 ears (**Figure 1** and **Figure 2**). With this technique, in combination with immunolabeling for CtBP2 (a marker for presynaptic ribbons) and GluA2 (a marker for post synaptic terminals), counting IHC/auditory nerve synapses using confocal images under Z projections with 0.25 µm intervals, based on the size of mouse ribbon synapses, is possible[21]. This is consistent with published results[4,6,13]. Surface preparations of CBA/J mice (without treatment) at the age of 10–12 weeks immunolabeled with CtBP2 (red) and GluA2 (green) showed that both presynaptic ribbons and post synaptic terminals are located below the IHC nuclei and are juxtaposed, indicating functional synapses (**Figure 3**)[6,13].

By immunolabeling surface preparations with different antibodies, assessment of molecular signaling and structure in sensory hair cells is possible. For example, **Figure 4** shows the results for immunolabeling for myosin VIIa and counterstaining with phalloidin and 4',6-diamidino-2-phenylindole (DAPI). Parallel immunolabeling experiments with and without the cell and tissue adhesive using identical solutions have been conducted to assess if the adhesive used interferes

with the immunoreactions. Cochlear surface preparations were immunolabeled with myosin VIIa and counterstained with phalloidin. Confocal images were taken with a 63x magnification lens under identical conditions and equal parameter settings for laser gains and photomultiplier tube (PMT) gains. There was no difference in immunoreactions or uniformity with and without the cell and tissue adhesive (**Figure 5**). Using different fixatives, surface preparations have provided the basis for scanning electron microscopy (SEM) images for visualization of cochlear stereocilia[9]. C57BL/6J mice (without treatment) at the age of 6–8 weeks show well-organized V-shape stereocilia of OHCs in three rows (**Figure 6**). Additionally, cochlear surface preparations have been used to determine the pattern of expression of a report gene (i.e., GFP) and confirm successful transduction and identify transduced cell types.

FIGURE AND TABLE LEGENDS:

Figure 1: Depiction of the steps for adult mouse surface preparations. (**a**) The cochlear bony capsule of the temporal bone faces up. The red line indicates the first cut to separate the apical turn. RW = round window, OW = oval window. (**b**) The apex is separated from the cochlea as indicated by the arrow. The red line between round window and oval window indicates the second cut made in the cochlea. (**c**) The cochlear portion that contains the middle, basal, and hook regions is separated from the vestibular portion. (**d**) The cochlear portion is situated facing up. The red line indicates the third cut, directed towards the end from which the apical section was removed. (**e**) The arrow indicates the gap left after the completion of the third cut. (**f**) This image shows the middle region. (**g**) This image shows the combined basal and hook regions. The vertical red line indicates the cut between the modiolus and hook region. The cut between the basal and hook regions is indicated by the other red line. (**h**) Basal and hook regions are separated as indicated by the arrows. (**i**) The middle region of the bony capsule is cut as indicated by the red line. (**j**) The image shows the middle region after one side of the bony capsule is removed. (**k**) This image shows the middle region after completion of dissection. (**l**) All four regions are completely dissected. (**m**) Cochlear turns affixed to a 10 mm round coverslip transferred to a four-well Petri dish with the four regions as indicated. All images were taken under a stereo-dissection microscope at 1.2x, 2.5x, and 0.6x magnifications. Scale bars are indicated in each image.

Figure 2: Location of the major cuts. The entire cochlear bony capsule was removed. The locations of the major cuts are indicated. 1) The location where the apical turn is cut. 2) The site where the middle turn is separated. 3) The critical cut to remove the modiolus. 4) The line to divide the basal and hook regions. Scale bar = 0.5 mm.

Figure 3: Confocal images reveal immunolabeling for CtBP2 and GluA2 on adult mouse surface preparations. The apical, middle, and basal regions are labelled. The lower magnification confocal images were taken with a 10x lens. Red = CtBP2, green = GluA2. Scale bar = 100 µm. Confocal images of 5-, 16-, and 32-kHz regions were taken with a 63x lens. Enlarged views of the areas indicated by the white rectangles in the apex, middle, and base portions. Scale bar = 10 µm.

Figure 4: Confocal images reveal immunolabeling of sensory hair cells of a surface preparation from the 32-kHz region. All images were merged Z-stack projections. Phalloidin (green) stained the structure of the three rows of OHCs and one row of IHCs. Myosin VIIa (red) immunolabeled three rows of OHCs and one row of IHCs. DAPI-stained hair cell nuclei. Merged confocal images were reconstructed for side views of sensory hair cells. Scale bar = 10 µm.

Figure 5: Cochlear surface preparations with and without the cell and tissue adhesive were processed in parallel, immunolabeled for myosin VII (red), and counterstained with phalloidin (green) using identical solutions. The cell and tissue adhesive used does not interfere with immunoreactions. Confocal images were taken with a 63x magnification lens under identical conditions and equal parameter settings. Images were taken from the 32-kHz region. Immunolabeling for myosin VIIa and staining for phalloidin in OHCs was similar with and without the cell and tissue adhesive. Scale bar = 10 µm.

Figure 6: Scanning electron microscopy shows three rows of OHC stereocilia from C57BL/6J mice. Images were taken from the middle region. (**a**) A low-magnification view of three rows of OHCs. (**b**) Enlarged images show three rows of well-organized OHC stereocilia that appear in "V" shapes. Scale bar = 10 µm.

Table 1: Mapping of the CBA/J mouse cochlear frequency sensitivity as a function of distance from the apex according to Muller[1] and Viberg and Canlon[2].

DISCUSSION:

Cochlear microdissection of whole-mount surface preparations in combination with immunolabeling provides a basic tool for investigation of inner ear pathologies and molecular mechanisms. This modified adult mouse cochlear dissection method using the cell and tissue adhesive simplifies this difficult procedure.

Although this modified cochlear surface preparation method is relatively easy and accessible, it still requires practice in order to achieve proficiency. To make the correct cuts, the dissector needs careful concentration. Because the cochlear sensory hair cells in the basal turn are so close to the spiral limbus, it is difficult to fully remove the limbus tissue to allow the surface preparation to lie completely flat, but confocal Z-projections can compensate for this issue. Additionally, the hook region of the cochlea is still the most difficult portion to dissect. Separations between OHCs and IHCs of the hook region may occur. The hook region displays large anatomic variations in humans and is important for bone-conduction hearing[22,23]. Based on the cochlear frequency mapping reported by Muller[1] and Viberg and Canlon[2], the hook region, beginning around 4.7 mm from the apex, corresponds to sensitivity to 48-kHz tones and higher (**Table 1**), whereas auditory functional assessments of acquired hearing loss in mice, including noise-induced, aminoglycoside-induced hearing loss, and age-related hearing loss, are generally measured at 8, 16, and 32 kHz with auditory brainstem response (ABR)[5–7,9,24] and from 6–45 kHz with distortion product otoacoustic emission (DPOAE)[25]. In agreement with Montgomery et al, distortion of the epithelium is not commonly seen when the spiral is divided into 3–4 pieces[19].

The modified method presented here involves dissecting the cochlear spiral into only four pieces (apex, middle, basal turns, and the hook region), whereas the Eaton-Peabody technique produces six pieces[18]. The Eaton-Peabody technique starts with a cochlear bisection, which avoids making tangential cuts through the epithelium to first separate the apical turn from the rest of the spiral, as described in this modified technique. By producing smaller pieces, the Eaton-Peabody technique is designed to minimize the flattening required to view a large piece with an immersion objective. In fact, the larger portions of tissue facilitate measurement of frequency mapping from the apical to the basal turn, in line with Montgomery et al.[19]. Additionally, a difference between this method and Montgomery et al.[19] is that the modified cochlear dissection method described here employs a scalpel for most cuts and only one step is done with scissors (i.e., the separation of basal and hook regions as illustrated in the third cut in **Figure 2**), whereas Montgomery et al.[19] used scissors with a silicone elastomer-coated dissection dish for surface preparations. To avoid distortion of the tissue, disconnection of the basilar membrane to remove the modiolus is critical.

This protocol uses a cell and tissue adhesive (**Table of Materials**) for adhesion of the pieces of cochlear epithelium to the 10 mm round coverslip for immunolabeling or immunohistochemistry, which makes the processes more convenient and avoids loss of epithelium tissues during the multiple washes of immunolabeling procedures. Cell and tissue adhesive is a formulation of polyphenolic proteins that adheres to cells and tissues and has been widely used in many common in vitro techniques, including immunohistochemistry, in situ hybridization, and immunoassays[20]. Consistent with the notion that the cell and tissue adhesive does not interfere with immunoreactions, parallel immunolabeling of myosin VIIa with surface preparations shows no difference of immunoreactions or uniformity with and without the cell and tissue adhesive. Comparing these three dissection methods (Eaton-Peabody Laboratories, Montgomery et al.[19], and the modified method described), the graduate students in the lab agree that this modified method using the cell and tissue adhesive is easier to learn and master.

In summary, producing whole-mount adult mouse surface preparations is a basic skill for evaluation of cochlear pathologies. The described modified protocol for adult mice surface preparations simplifies this difficult procedure.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1-Dissection images 07-24-19.tif

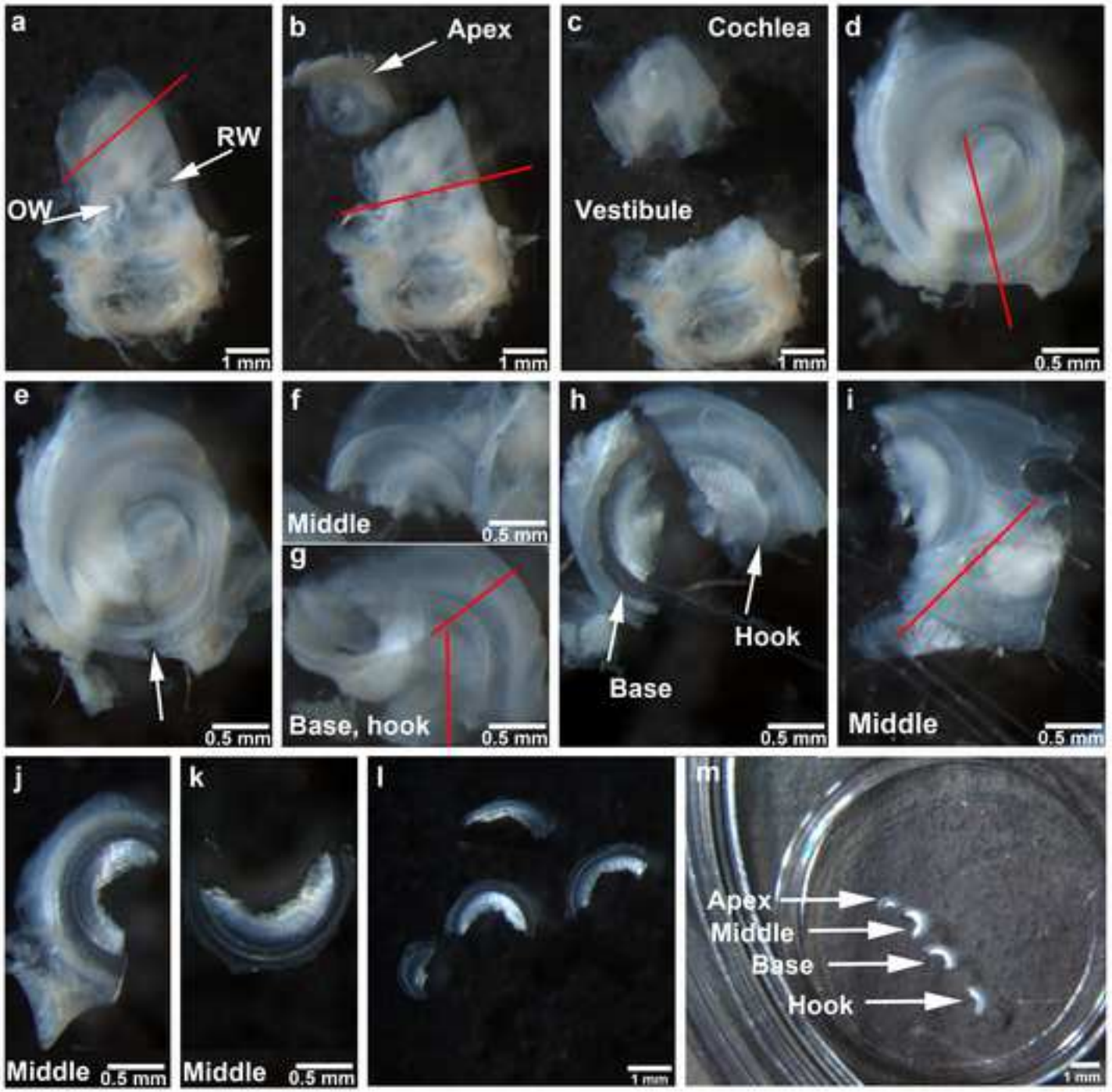

Figure 2

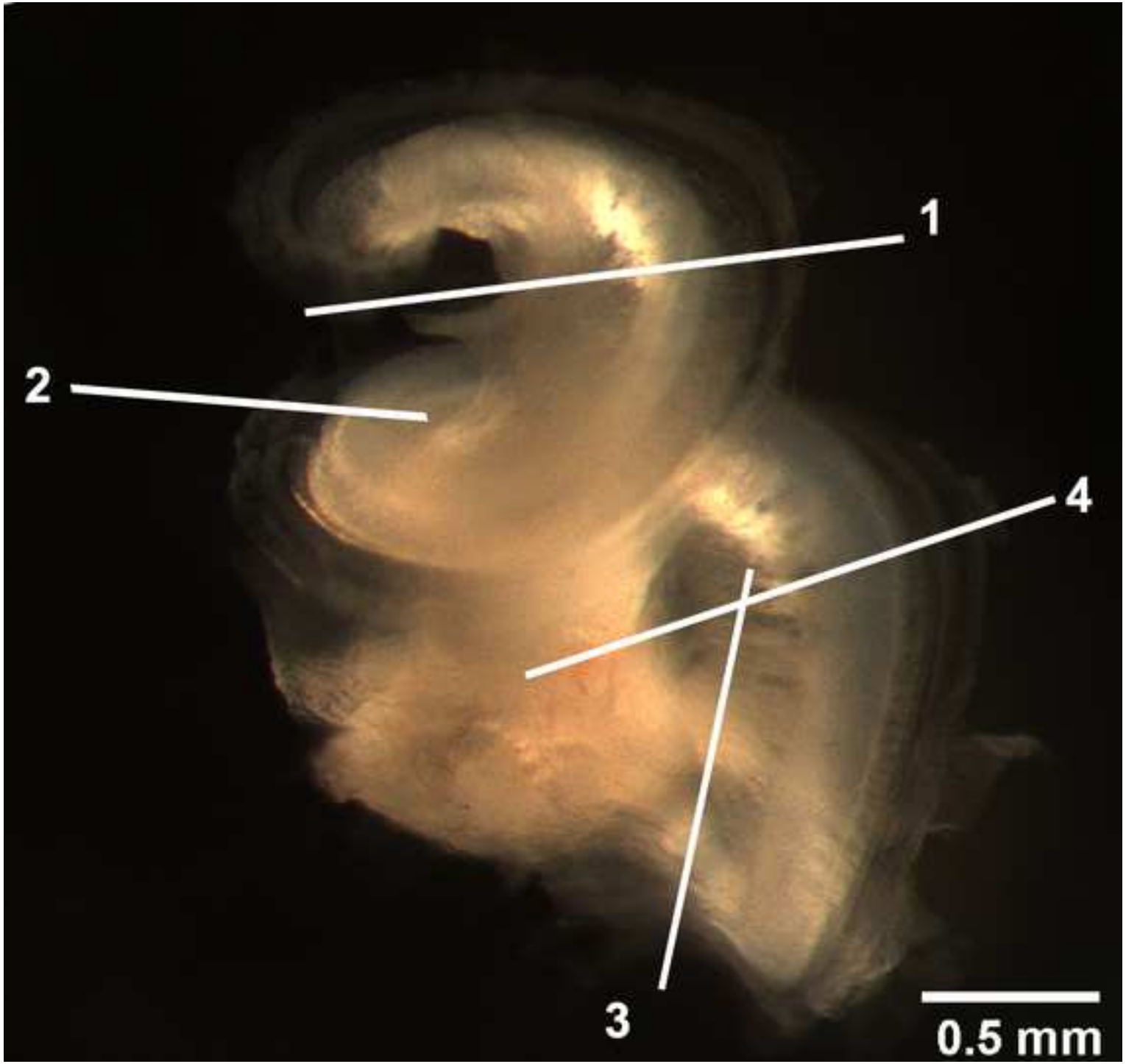

Figure 3

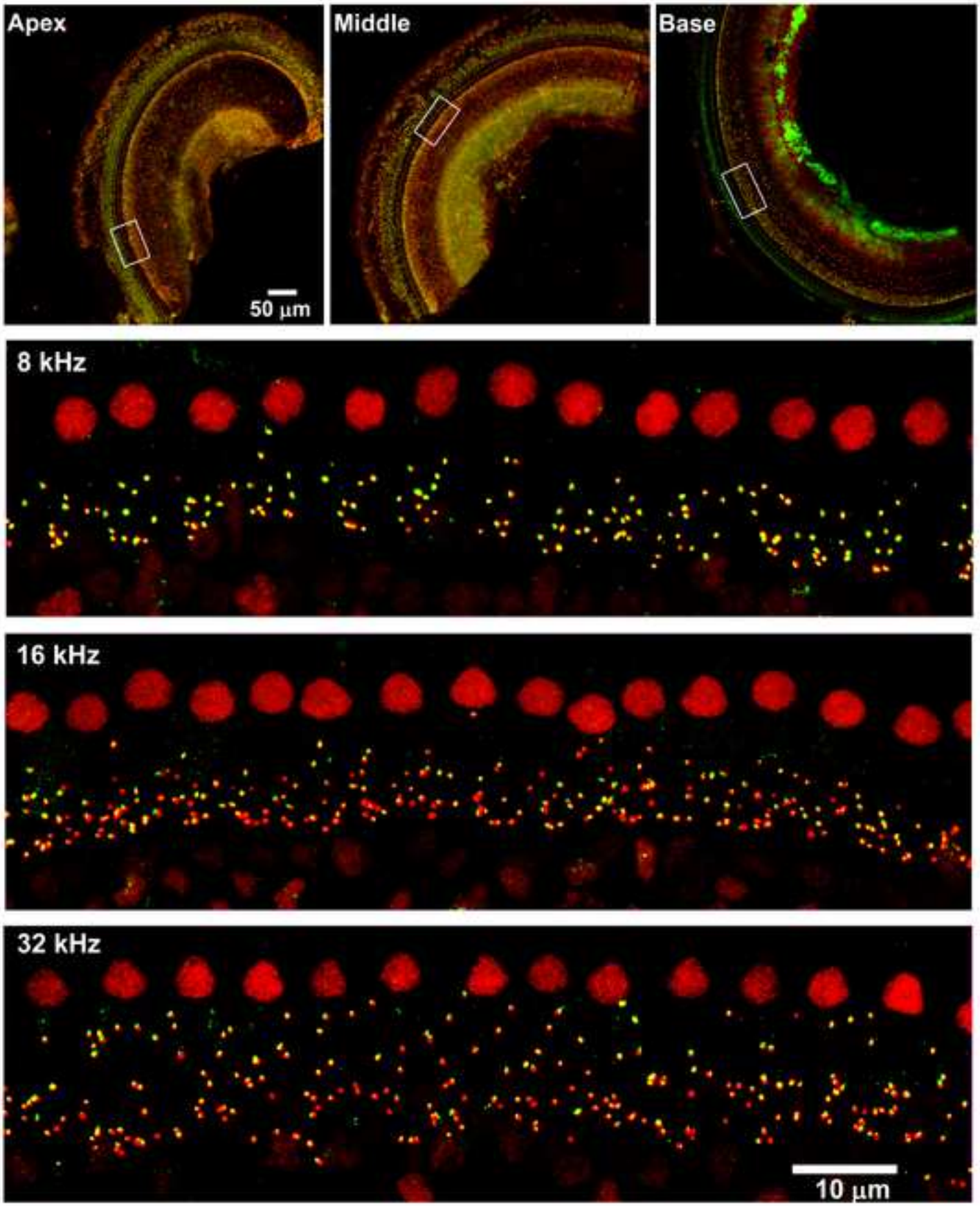

Figure 4

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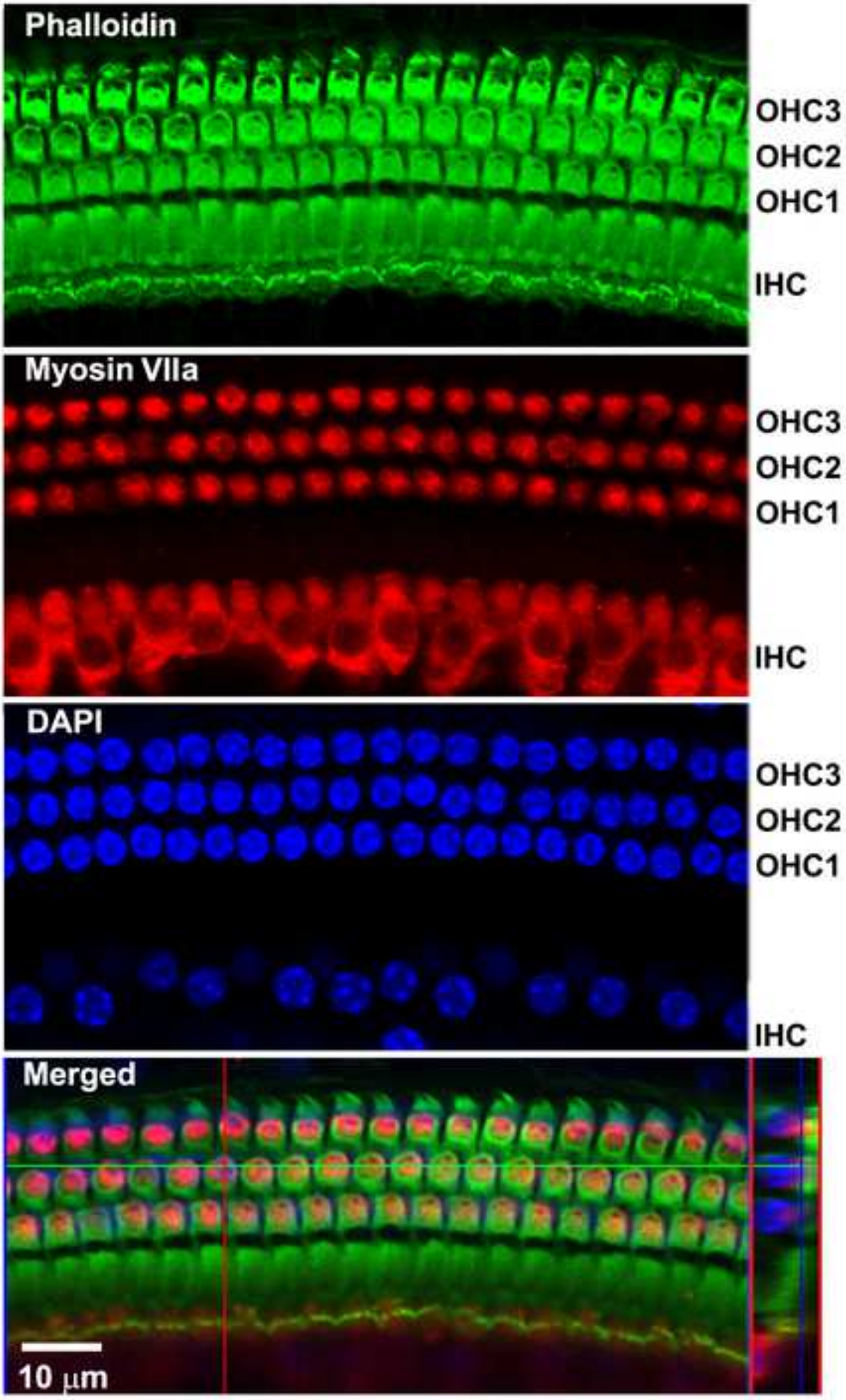

Figure 5

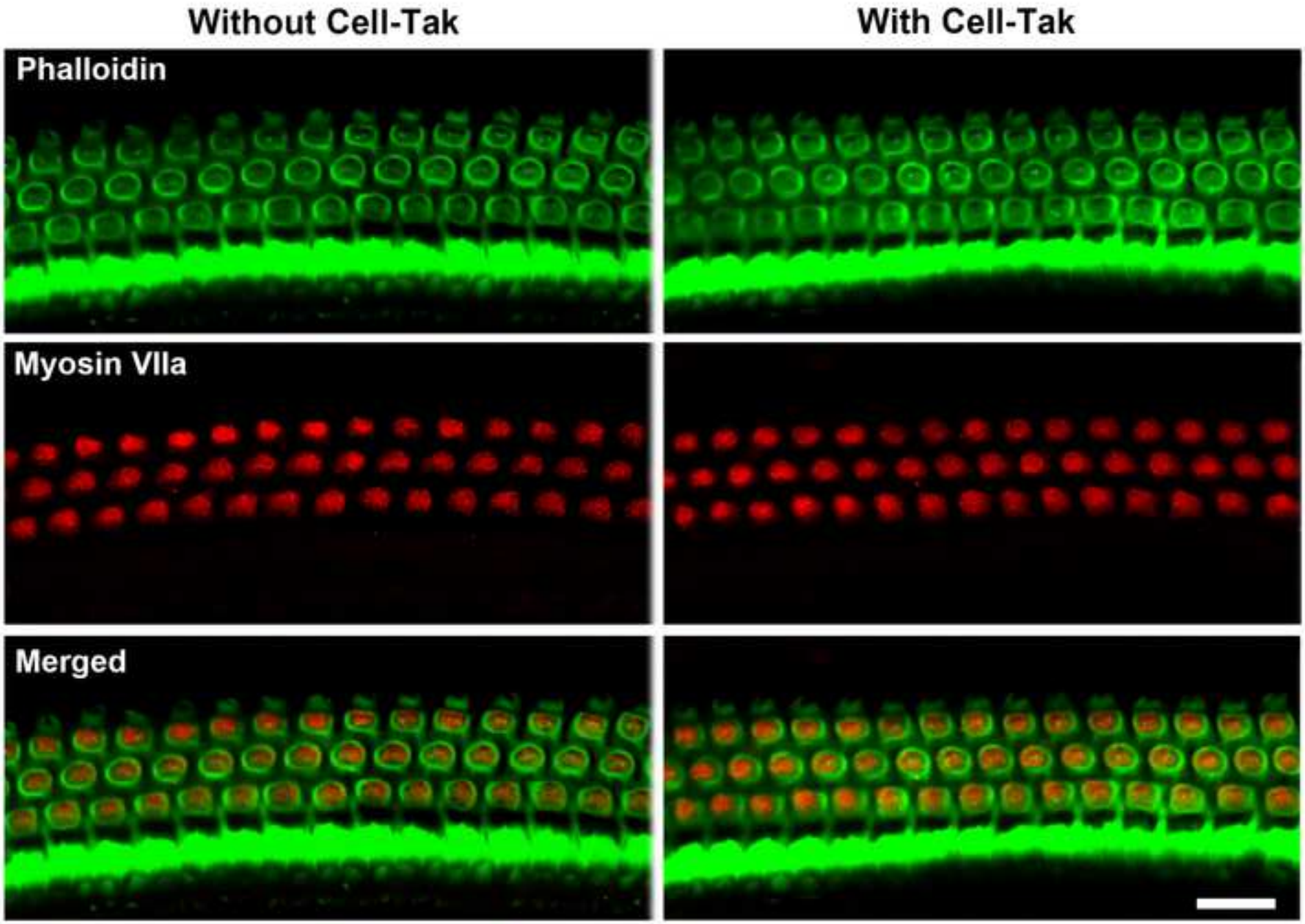

Figure 6

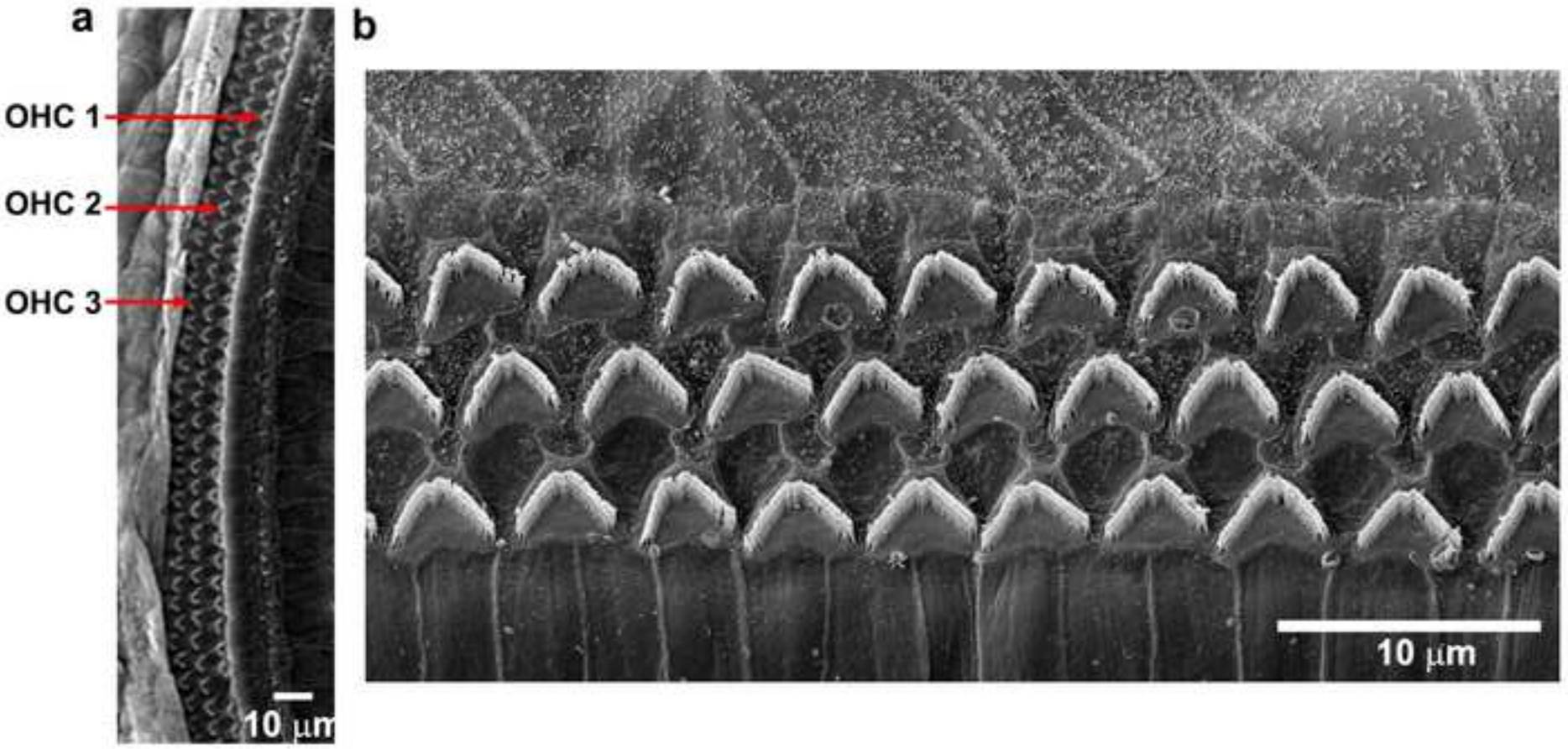

Table 1

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Name						
Distance from the apex (mm)	0.4	1	2.4	3.3	3.9	4.7
Distance from the apex (%)	7.7	18	43	54	68	82
Frequencies (kHz)	6	8	16	22	32	48

5.7
100

Name of Material/ Equipment	Company
10-mm Rund Coverslips	Microscopy products for science and industry
Alexa Fluor 488 Goat Anti-mouse IgG2	Thermo Fisher Scientific
Alexa Fluor 488 Phalloidin	Thermo Fisher Scientific
Alexa Fluor 594 Goat Anti-mouse IgG1	Thermo Fisher Scientific
Alexa Fluor 594 Goat Anti-rabbit IgG (H+L)	Thermo Fisher Scientific
Carboard Micro Slide Trays	Fisher Scientific
Cell-Tak	BD Biosciences
Corning Petri Dishes	Fisher Scientific
DAPI	Thermo Fisher Scientific
Dumont #5 Forceps	FST fine science tools
EDTA Disodium Salt	Sigma-Aldrich
Fluoro-gel with Tris Buffer	Electron Microscopy Sciences
Four-well Cell Culture Dishes	Greiner Bio-One
Goat Anti-myosin VIIa Antibody	Proteus Biosciences
Microscope Slides	Fisher Scientific
Mouse Anti-CtBP2 Antibody	BD Biosciences
Mouse Anti-Glu2R Antibody	Millipore
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Phosphate Buffered Saline	Fisher Scientific
Scalpel	VWR
Triton X-100	Sigma-Aldrich
Vannas Spring Scissors	Fine Science Tools

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A-21131
A12379
A-21125
A11012
12-587-10
354240
353004
62247
11251-20
E5134
17985-10
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12-544-7
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MAB397
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Xiaoyan Cao, PhD.
Review Editor
JoVE

July 28, 2019

Dear Dr. Cao,

I sincerely appreciate the opportunity for resubmission of our manuscript entitled "Cochlear surface preparations in the adult mouse" (JoVE60299). I also appreciate the editor's detailed comments and reviewers' critiques for revision of the manuscript. The following are point-by point responses to the comments. All changes made in the revised manuscript are highlighted.

Editorial comments:

1. *Regarding proofreading of the manuscript*: This manuscript has been proofread by a professional scientific editor.
2. *Each author's institutional email address* is added.
3. *Keywords*: Eight key terms have been added: cochlear surface preparation, whole mount dissection, sensory hair cells, cochlear ribbon synapses, adult mice, immunolabeling, immunostaining, immunohistochemistry
4. *Summary section*: The summary section has been added before the abstract section.
5. *Superscript number for reference in lines 84-85, 88-89*: Done
6. *Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.*

The following paragraph has been added to the introduction:

"Unlike immunohistochemistry using paraffin sections or cryosections to obtain small cross-sectional portions of the cochlea that contain three outer hair cells (OHCs) and one inner

hair cell (IHC) on each section, cochlear surface preparations allow the advantage of visualization of the entire length of the organ of Corti for counting sensory hair cells and ribbon synapses and immunolabeling sensory hair cells corresponding to specific functional frequencies. Table 1 shows the mapping of hearing frequencies as a function of distance along the length of the cochlear spiral in CBA/J mouse according to papers from Mueller (2005) and Viberg and Canlon (2004) (Viberg & Canlon, 2004; Muller *et al.*, 2005).

Cochlear surface preparations have been widely used for investigation of cochlear pathologies(Jiang *et al.*, 2006; Weber *et al.*, 2008; Kujawa & Liberman, 2009; Chen *et al.*, 2012; Wan *et al.*, 2014; Yuan *et al.*, 2015; Hill *et al.*, 2016). Micro-dissection of the cochlea is an essential and critical step for cochlear surface preparations. However, dissecting mouse cochleae is a technical challenge and requires a lot of practice. Here, we present a modified cochlear surface preparation method for use in adult mouse cochleae that requires decalcification and use cell-Tak to adhere the pieces of cochlear epithelia to the 10-mm round cover slip for procedures of immunolabeling. Cell-Tak has been widely used as an adhesive for cells or tissues for immunohistochemistry. Based on our experience, we believe this modified cochlear microdissection method is relative simple compared to reported in the literature."

7. *Remove commercial names*: Since all companies' names were listed in the materials section and that entire section was deleted, there are no longer any commercial names in the revised manuscript.
8. *Remove materials section*: Done
9. *Protocols should be described in the imperative tense; remove the discussion about the protocol in the discussion section:* We have changed the verb tense used in the protocol to the imperative tense and removed sentences related to the protocol from the discussion.
10. *Adding more details to the protocol step:* We have added details into each step based on the guidelines from 11–12.

11. *Section 1: Please specify the age, gender and type of animal used here. Is the mouse anesthetized or sacrificed before extracting bones? Please specify how and also specify all surgical tools used throughout the protocol.*

    We have revised ethics statement as following:

    Procedures involving male adult CBA/J mice at the ages of 10-12 weeks and C57BL/6J mice at the ages of 6-8 weeks were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. Mice were decapitated after the animal no longer responds to painful stimuli, such as toe pinch.

    The section 1 has also been revised as following:

    1) Decapitate the mouse immediately post-mortem with surgical scissors (17 cm long) and cut the skull bone with scissors from the posterior aspect forward along the center line of the skull after exposing the skull bone by pulling the skin anteriorly.
    2) Remove brain tissue using forceps and manually remove the temporal bones with the thumb and index figure for mice 2 months of age or older. Use small surgical scissors (11 cm long) to cut out the temporal bone of mice younger than 2 months old.
    3) Put the temporal bone into a Petri dish (30 mm in diameter) containing ice-cold fresh 4% paraformaldehyde solution (PFA) dissolved in phosphate buffered saline (PBS), pH 7.4.

12. *Please simplify the Protocol so that individual steps contain only 2-3 actions per step with a maximum of 4 sentences per step. Use sub-steps as necessary*:

    We have simplified the protocol following the editor's instructions.

13. *Discussion: Please describe any limitations of the method*. We have an almost completely revised discussion.

    Following are the revised portions of our discussion.

    "Although our modified cochlear surface preparation methods are relatively easier and accessible, they still require practice in order to achieve proficiency. To make the correct cuts, the

dissector needs careful concentration. Since the cochlear sensory hair cells in the basal turn are so close to the spiral limbus, it is unlikely to fully remove the limbus tissue to allow the surface preparation to lie completely flat, but confocal Z-projections can take care of this issue. Additionally, the hook region of cochlea is still the most difficult portion to dissect. Separations between OHCs and IHCs of the hook region may occur. The hook region displays large anatomic variations in humans and holds importance for bone-conduction hearing (Atturo *et al.*, 2014; Kim *et al.*, 2014). Based on cochlear frequency mapping reported by Mueller, Viberg and Canlon (Viberg & Canlon, 2004; Muller *et al.*, 2005), the hook region beginning 4.7 mm from the apex corresponds to sensitivity to 48-kHz tones and higher (Table 1), whereas auditory functional assessments of acquired hearing loss in mice, including noise-induced, aminoglycoside-induced hearing loss, and age-related hearing loss, are generally measured at 8, 16, and 32 kHz with auditory brainstem response (ABR) (Chen *et al.*, 2012; Zheng *et al.*, 2014; Yuan *et al.*, 2015; Hill *et al.*, 2016; Wang *et al.*, 2018) and from 6–45 kHz with distortion product otoacoustic emission (DPOAE) (Brown *et al.*, 2017). In agreement with a report from Dr. Cox's group, distortion of the epithelium is not commonly seen when the spiral is divided into 4 pieces (Montgomery & Cox, 2016).

Indeed, our modified methods involve dissecting the cochlear spiral into only 4 pieces (apex, middle, basal turns, and the hook region), whereas the Eaton-Peabody technique produces 6 pieces. The Eaton-Peabody strategy starts with a cochlear bisection, which avoids making tangential cuts through the epithelium to first separate apical turn from the rest of the spiral, as described in our modified techniques. By producing smaller pieces, the Eaton-Peabody technique is designed to minimize the flattening required to view a large piece with an immersion objective. In fact, the larger portions of tissue facilitate measurement of frequency mapping from the apical to basal turn, in line with the report from Dr. Cox's group (Montgomery & Cox, 2016). Additionally, a difference between our method and the report from Dr. Cox's group is that our modified cochlear dissection methods employs a scalpel for most cuts and only one step is down with scissors

(separation of basal and hook regions as illustrated in figure 2, the third cut 3), whereas Dr, Cox's group used scissors with a silicone elastomer-coated dissection dish for surface preparations. To avoid distortion of the tissue, disconnection of the basilar membrane to remove the modiolus is critical.

We use cell-Tak for adhesion of the pieces of cochlear epithelium to the 10-mm round cover slip for the procedures of immunolabeling or immunohistochemistry, which makes such processes more convenient and avoids loss of epithelium tissues during the multiple washes of immunolabeling procedures. Cell-Tak for adhesion of cells and tissues is a formulation of "polyphenolic proteins" and has been widely used in a number of common *in-vitro* techniques, including immunohistochemistry, *in-situ* hybridization, and immunoassays based on the description from producers (BD Biosciences). Consistent with the notion that cell-tak does not interfere with immunoreaction, parallel immunolabeling of myosin VIIa with surface preparations shows no difference of immunoreactions or uniformity with and without cell-Tak.

By comparison of these three dissection methods (Eaton-Peabody Laboratories, that of Dr. Cox's Lab, and our modified methods), the graduate students in the lab agree that our modified method in combination with cell-Tak is easier to learn and master."

14. *References:* All references follow the standards of JoVE.
15. *Figure 1:* Scale bars for all images have been added in the lower right corner and defined in the figure legend.
16. *Table of Materials*: All information for relevant materials has been added into the material table.

Reviewers' comments:

Reviewer #1

1. *The trade-offs between the Eaton-Peabody and our modified methods should be acknowledged.*

We added a paragraph to the introduction to describe the advantages of cochlear surface preparations. Please see the response to editorial comment #6 above.

We also revised the discussion regards to the differences between cochlear surface preparations from our modified methods and methods from Eaton-Peabody laboratories and Dr. Cox's group. Please see the response to editorial comments #13 above.

2. *Regarding the narrative presented being very difficult to follow because numerous anatomical terms are undefined (e.g. modiolus, lateral wall, hook), because the directionality terms are ambiguous (e.g. top vs. bottom, up vs. down and front vs. back), and because not enough textual detail is provided:*

We have defined anatomical terms such as modiolus, lateral wall, and hook before we use these terms (see below). We also added pictorial references to top vs. bottom, up vs. down, and front vs. back when used to describe the directionality. Based on the editorial suggestion, we have revised the protocol using the imperative tense and to contain 2–3 actions per step. We believe the revised manuscript is concise and clearer.

"The modiolus is the conical-shaped central axis of the cochlea that consists of spongy bone and cochlear nerve as well as the spiral ganglion."

"The lateral wall of the cochlear duct is formed by the spiral ligament and the stria vascularis."

"The hook region of the cochlea is the end of the cochlear epithelium corresponding to sensitivity to 48-kHz tones and higher in mice."

3. There is a major omission on how cochlear location is mapped into frequency in this approach.

We have added table 1 to illustrate mapping of the CBA/J mouse cochlear frequency sensitivity as a function of distance from the apex according to Mueller (2005), Viberg and Canlon (2004)(Viberg & Canlon, 2004; Muller *et al.*, 2005).

4. *Images are of low quality which may be due to the preparation of the pdf file.*

   Yes. In fact, all of the images are with resolution of 300 dpi.

Reviewer #2:

The attachment of the preparation onto the glass coated with cell-Tak will result in an area (the attached area of the preparation) to be either not stained or unevenly stained. Since the bottom end of the preparation is in contact with the cell-Tak the authors have no control over how much of the cell-Tak that will be interfering with the base of the surface preparation.

Based on the description from BD Biosciences, cell-Tak for adhesion of cells and tissues is a formulation of "polyphenolic proteins" and has been used in a number of common *in-vitro* techniques, including immunohistochemistry, *in-situ* hybridization, and immunoassays. We also conduct parallel immunolabeling experiments with and without cell-Tak. Cochlear surface preparations were immunolabeled with myosin VIIa, counterstained with phalloidin. Our results showed no difference of immunoreactions or uniformity with and without cell-Tak (Figure 4), indicating that cell-Tak does not interfere with immunolabeling.

Reviewer #3:

1. *Ribbon synapse staining is very difficult. If the author can provide more detailed information on this protocol, this manuscript will be improved.*

We followed the protocol of immunolabeling of ribbon synapses using CtBP2 and GluA2 antibodies from Dr. Corfas's lab at the Kresge Hearing Research Institute, University of Michigan (Wan *et al.*, 2014). We have added this protocol into the revised manuscript.

2. *What is the interval distance between slices when the author took images using z-stack, especially for ribbon synapses?*

Based on the size of mouse ribbon synapses(Nouvian *et al.*, 2006), we have tested interval distances from 0.1–0.25 µm for ribbon counting. The results from ribbon counting with 0.25-µm intervals is similar as a 0.1-µm intervals. We therefore routinely use a 0.25-µm interval distance

for the Z-stack projections, which is in agreement with the reports from Drs. Kujawa and Liberman's group (Kujawa & Liberman, 2009).

3. *DAPI was marked in the nucleus image in figure3, however, in the legend, outer hair cell nuclei were stained by Hoechst 33342*. *More, all the nuclei can be stained using both Hoechst 33342 and DAPI. Thus "Outer hair cell nuclei" should be changed to "hair cell nuclei"*

DAPI was used for nuclei staining in these images. The legend has been changed to DAPI. We also changed "outer hair cell nuclei" to "hair cell nuclei".

Reviewer #4

1 *It is recommended that the authors add schematics as complemental images of Figure 1 to show the location of cuts*.

We have added figure 2 to show the location of cuts, since there is no space available to add it in figure 1.

2 *Figure 4 SEM. It is recommended to provide a low-magnification view of the sample.*

We have added a low-magnification view of the sample in figure 6a SEM (original figure 4).

3 *This method is a modified method. It is recommended that the authors briefly describe what specific modifications have been made and how these modifications make the method better.*

We have addressed this comment in the response above to editorial comment #13.

Reviewer #5

1 *The authors need to add these 2 sentences, "Anesthetize the mice with intraperitoneal injection of the (anesthetic used). Euthanize or decapitate the mouse only after the animal no longer responds to painful stimuli, such as toe pinch.*

We have added this information in the proper place.

2. *Minor changes for improvement of the manuscript:* I sincerely appreciate all detailed edits. Every suggested change has been incorporated in the revised manuscript.

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