Video Article

Dissection of the Auditory Bulla in Postnatal Mice: Isolation of the Middle Ear Bones and Histological Analysis

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Abstract

In most mammals, auditory ossicles in the middle ear, including the malleus, incus and stapes, are the smallest bones. In mice, a bony structure called the auditory bulla houses the ossicles, whereas the auditory capsule encloses the inner ear, namely the cochlea and semicircular canals. Murine ossicles are essential for hearing and thus of great interest to researchers in the field of otolaryngology, but their metabolism, development, and evolution are highly relevant to other fields. Altered bone metabolism can affect hearing function in adult mice, and various gene-deficient mice show changes in morphogenesis of auditory ossicles *in utero*. Although murine auditory ossicles are tiny, their manipulation is feasible if one understands their anatomical orientation and 3D structure. Here, we describe how to dissect the auditory bulla and capsule of postnatal mice and then isolate individual ossicles by removing part of the bulla. We also discuss how to embed the bulla and capsule in different orientations to generate paraffin or frozen sections suitable for preparation of longitudinal, horizontal, or frontal sections of the malleus. Finally, we enumerate anatomical differences between mouse and human auditory ossicles. These methods would be useful in analyzing pathological, developmental and evolutionary aspects of auditory ossicles and the middle ear in mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/55054/

Introduction

The three auditory ossicles of the middle ear, namely the malleus, incus, and stapes, form a mammalian-specific auditory chain that transmits sound from the tympanic membrane to the inner ear, or cochlea [1,2]. Hearing function can be evaluated in mice by measuring Auditory Brainstem Response (ABR) thresholds [3-6], and vibration of the malleus behind the tympanic membrane can be monitored using Laser Doppler Vibrometry (LDV) [7]. By combining ABR, LDV, and Distortion Product Otoacoustic Emission (DPOAE) measurements, conductive hearing loss can be discriminated from sensorineural impairment [8].

Animal models of ear conditions are needed, given the importance of hearing and ear health to the well-being of patients of all ages. For example, otitis media is an extremely common ear infection seen in human infants and children, and severe, acute otitis media and its complications can occur if the condition is not treated with appropriate antimicrobials [9]. Mouse models of otitis media could prove useful in understanding the pathogenesis and in developing treatments [10,11].

Murine ossicles, which (except for the goniale part of the malleus) are formed by endochondral ossification [12,13], are highly relevant to the study of bone metabolism and morphogenesis. First, their small size allows high-resolution analysis of bones with an intact periosteum using X-ray or fluorescence microscopy [14]. Second, aberrant bone metabolism, such as excessive or deficient bone resorption, or impaired interactions among bone cells [15], can be analyzed as a potential contributor to hearing loss [3,4,7]. Third, abnormal ossicle morphogenesis is reported in several gene-deficient mice, such as animals lacking *Hoxa2* [16-19], *Msx1* [20-22], *Prrx1* [23], *Goosecoid* (*Gsc*) [24,25], *Bapx1* [13], *Tshz1* [26], *Dusp6* (*Mkp3*) [27], *Noggin* (*Nog*) [28], *Fgfr1* [29], thyroid hormone receptors (*Thra*, *Thrb*) [5], *Bcl2* [30] and others [1,31], or in mice overexpressing *Hoxa2* [32]. Finally, despite their small size, structures associated with ossicles such as muscles [33] and joints [34,35] are accessible.

Mouse ossicles are smaller than human ossicles, but it is noteworthy that the mouse middle ear is not a miniature version of its human counterpart. For example, in mice, the stapedial artery, which passes through the ring of the stapes, persists throughout life [36], whereas in humans, the embryonic stapedial artery disappears during gestation. In addition, the morphology of the mouse malleus differs from that of the human bone (see **Figure 6**). In mice, the auditory (tympanic) bulla encloses the air-filled middle ear cavity, whereas in humans, mastoid air cells composed of trabecular bone in the temporal bone houses the ossicles rather than a bulla [37]. In both species, the auditory capsule (otic capsule, bony labyrinth) encloses the cochlea and semicircular canals of the inner ear. Comparative and evolutionary biology of the middle ear has been extensively reviewed [38-40].

The protocol provided below first describes how to dissect out the auditory bulla and capsule, which consist primarily of the middle ear and inner ear, respectively. This protocol also demonstrates how to isolate the malleus, incus and stapes from the auditory bulla. Finally, it shows how to orient the auditory bulla and capsule for embedding in preparation for tissue sectioning of auditory ossicles.

Protocol

All animal procedures performed in this study are approved by the Keio University Institutional Animal Care and Use Committee (IACUC - approval number: 09221) and follow the Institutional Guidelines on Animal Experimentation at Keio University for the use of animals in research. Human specimens were isolated from a cadaver donated to the Department of Anatomy, Keio University School of Medicine, and were used in accordance with institutional regulations.

1. Isolation of Auditory Bulla and Capsule

1. Euthanize mice in a jar containing a platform above paper towels soaked in isoflurane or sevoflurane until respiratory ventilation ceases for more than a min and then perform cervical dislocation. Be careful to avoid direct contact of mice with the soaked paper towels.
2. Make a small transverse incision at the dorsal side of the neck and pull skin apart toward the head and tail using both hands to expose underlying neck muscle tissue.
3. Decapitate mice at the cervical region using 14 cm sharp surgical scissors.
4. Peel skin completely towards the nose. Cut off all skin together with the snout and incisors.
5. Insert scissors into the mouth and cut masseter muscles on both sides.
6. Open the jaw carefully and remove the tongue and lower jaw together.
7. Using sharp scissors, split skull and skull base into two halves along the midsagittal plane (**Figure 1A, B**).
8. Using forceps, remove the cerebral and cerebellar hemispheres and the brainstem. The auditory bulla and capsule are located lateral to the cerebellum and brainstem. Note that the auditory bulla is further lateral to the auditory capsule (**Figure 1C, D**).
9. Dissect out the bulla and capsule with the surrounding skull bone (**Figure 1E**).
10. Transfer the specimen to a dish containing Phosphate-buffered Saline (PBS) pH 7.4 at RT.
11. Under a binocular dissecting microscope, use forceps to pull apart the surrounding bones and scissors to cut the loosened boundary around the bulla and capsule (**Figure 1F**). The surrounding bones removed are the basioccipital (ventral border), exoccipital (ventro-posterior border), supraoccipital (posterior border), interparietal, parietal (dorsal border), squamosal (dorso-anterior border), alisphenoid (anterior border), and basisphenoid (antero-ventral border) bones. Note that the styliform process (Sp), which supports the tympanic opening of the Eustachian tube [41], is distinct from the styloid process of the temporal bone.

2. Isolation of Auditory Ossicles: Malleus, Incus and Stapes

1. **Malleus**
   1. Using both small scissors and forceps, remove the part of the external auditory canal lateral to the sulcus tympanicus so that the tympanic membrane is visible (**Figure 2A, B**).
   2. Remove part of the tympanic membrane and tympanic bone near the malleal processus brevis (orbicular apophysis, see Discussion), both at the ventral (dotted) and posterior (#) walls (**Figure 2C**). The malleus and tensor tympani muscle should now be exposed (**Figure 2D, E**).
   3. Lift the malleus (**Figure 2F**) and cut the tensor tympani muscle with the beveled edge of a 27 G needle (**Figure 2G**). Note that the malleal manubrium firmly attaches to the tympanic membrane, as is seen in other mammals.
   4. Detach the tympanic membrane carefully from the manubrium, which is fragile. Remove the tympanic bone to reveal the three auditory ossicles.
   5. Dislocate the malleus from the incus at the ossicular joint (**Figure 2H**).
   6. Isolate the malleus by fracturing the anterior process at the goniale.

2. **Incus and Stapes**
   1. Isolate the incus by cutting off the posterior ligament of the incus at the short crus (**Figure 3A**).
   2. Isolate the stapes by cutting off the stapedial artery near the stapes with the beveled edge of a 27 G needle (**Figure 3B, C**). If necessary, cut the tendon of the stapedial muscle at the muscular process of the stapes with the needle.
   3. Insert a sewing needle (or a marking pin) into the obturator foramen of the stapes and lift up the stapes. After removing the stapes, the oval window opening should be clearly visible (**Figure 3D**).

3. Embedding of Auditory Bulla and Capsule

1. **Preparation for embedding in paraffin blocks**
   1. Isolate the bulla and capsule as described in Section 1.
   2. Cut the anterior end of the bulla (the styliform process) off with scissors, immerse the bulla and capsule in 4% paraformaldehyde (PFA) in PBS at 4 °C, and allow fixative to enter into the bulla. If air becomes trapped in the bulla, remove it with a needle and syringe. Leave the bulla and capsule in the fixative at 4 °C O/N on a tube rotator.
      Caution: PFA is toxic and should be handled carefully.
   3. Wash once with PBS.
   4. Decalcify bulla and capsule for a week at 4 °C in 10% ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-2Na), 100 mM Tris base, pH 7.0, in a 2 mL tube. Change the buffer every other day.

5. Wash once with PBS. Specimens can be stored in 70% ethanol in water at 4 °C. Optionally, transfer to 70% ethanol through a graded alcohol series (30%, 50%, 70% in water).
6. On a tissue processor, dehydrate specimens in a graded series of ethanol solutions (70%, 2x 95%, 3x 100%, each 1 h), clear in xylene (4x, each 1 h at 40 °C), and infiltrate specimens with molten paraffin wax[42]. Optionally, substitute xylene with commercial tissue clearing solution (*e.g.*, Histo-Clear).
7. Unload specimens from the processor, and remove them from their cassettes.
8. On a tissue embedding console system, place specimens into molds filled with molten paraffin wax. Proceed to embedding (Section 4).

2. **Preparation for embedding in frozen blocks (Kawamoto's film method)[43]**
    1. Isolate the bulla and capsule as described in Section 1.
    2. Cut the anterior end of the bulla (the styliform process) off with scissors, immerse the bulla and capsule in fixative (2% or 1% PFA rather than 4% in PBS to preserve antigenicity) at 4 °C. If air is trapped in bulla, remove it using a needle and syringe. Leave the bulla and capsule in the fixative at 4 °C O/N on a tube rotator.
    3. Wash bulla and capsule quickly in PBS and immediately immerse in liquid cryo-embedding compound at 4 °C.
    4. Important: Remove air bubbles if any in the middle and outer ear through aspiration by a needle, and by adding the embedding compound with forceps. Proceed to embedding (Section 4).

4. Sample Orientation and Embedding

NOTE: The whole bulla and capsule must be arranged in a particular orientation during embedding to cut desired sections. The procedures outlined below are used to section the malleus in various orientations.

1. **Longitudinal (parasagittal) sectioning of the malleus**
    1. Put the lateral side of the bulla or external auditory meatus down in warm paraffin (or cryo-embedding compound). Adjust the orientation so that the neck and transversal lamina of the malleus are parallel to the horizontal bottom of the embedding dish (**Figure 4A - C**). Note that the tympanic membrane is inclined at an angle of approximately 30° to the vertical in the mouse head (**Figure 4A**; Figure 59 in Kampen[44]).
2. **Horizontal sectioning of the malleus**
    1. Place the dorsal crest horizontally in warm paraffin (or cryo-embedding compound). Adjust the orientation of the bulla and capsule so that the neck and transversal lamina of the malleus are perpendicular to the bottom of the embedding dish (**Figure 4D - F**).
3. **Frontal sectioning (cross sections) of manubrium and tympanic membrane [5]**
    1. Place the malleal manubrium in warm paraffin (or cryo-embedding compound) such that it is perpendicular to the bottom of the embedding dish.
4. Cool down the block to temperatures appropriate to harden paraffin wax on a tissue embedding console system (alternatively, use cryo-embedding compound in a dry ice/hexane bath).
5. Process tissue block and cut sections using routine procedures. For example, stain paraffin sections with hematoxylin and eosin (H&E), safranin O (for cartilage), or for Tartrate-resistant Acid Phosphatase (TRAP) activity (for osteoclasts) [3], or by immunohistochemistry. Undecalcified cryosections are suitable for bone labeling using fluorochromes [14], alizarin red staining for calcium, and immunofluorescence [42].

Representative Results

This protocol presents a method to isolate ossicles from the mouse auditory bulla. First, the bulla and capsule are dissected out as a single piece from the skull (**Figure 1**). The dissected bulla is then used to prepare the malleus (**Figure 2**) and the incus and stapes (**Figure 3**). Landmarks of the auditory bulla and capsule are the styliform process at the anterior end of the bulla, the dorsal crest, anterior semicircular canal, and the subarcuate fossa (**Figure 1F**). Microcomputed tomography (CT) imaging reveals ossicles in the auditory bulla as well as the optimal orientations for longitudinal and horizontal sectioning of those ossicles (**Figure 4**).

For longitudinal paraffin sectioning of the malleus, the bulla and capsule were decalcified in EDTA at 4 °C for one week, embedded in a paraffin block at the orientation shown in **Figure 4 A - C**, sectioned at 4 µm, and then stained using H&E. The malleus attached to the tympanic membrane in the auditory bulla revealed ongoing endochondral ossification at P14 (**Figure 5A**). To visualize new bone formation, calcein (30 µg/g bodyweight) was peritoneally injected into a P20 mouse, and bulla and capsule were isolated 24 h later at P21. The sample without decalcification was embedded frozen and then cryosectioned at 6 µm using an adhesive film based on the method of Kawamoto [43]. After nuclear staining with DAPI (4',6-diamidino-2-phenylindole), the section was observed under a fluorescence microscope. Calcein signals (green) revealed new bone formation in the malleus (m), bulla and capsule (**Figure 5B**). For horizontal sectioning of the malleus, the auditory bulla isolated from a 5-week-old mouse was embedded frozen without decalcification (for the orientation see **Figure 4D - F**), cryosectioned at 6 µm using the Kawamoto method, and stained using H&E. Horizontal sectioning of the malleal processus brevis (mPB) also shows the cochlea (**Figure 5C**).

A medial view of the right auditory ossicles isolated from a P31 mouse shows typical features of the mouse malleus, namely, the "gliding-seagull-wing-like" (or Persian sword-like [45]) manubrium, a prominent processus brevis (orbicular apophysis, see Discussion), and the transversal lamina (**Figure 6**). Note that the anterior process (processus anterior) was fractured in the dissection procedure around the goniale and was separated from the tympanic ring (ectotympanic). This representative sample exhibits an intact incudomalleolar joint between the malleus and incus, whereas the incudostapedial joint is dislocated. Tendinous insertions into the malleal and stapedial muscular processes are detectable (**Figure 6A**, asterisks).

Figure 6B compares mouse and human auditory ossicles at the same magnification. Species differences, other than size, include the following. The malleal manubrium is wing-like in mice but club-like in humans. The angle between the anatomical axis (or the axis of rotation, the line through the anterior process of the malleus and the short process of the incus) and the manubrium is much smaller in mice and the two are almost parallel, as opposed to nearly perpendicular in humans [6,46-48]. In human ossicles, vibrometric studies reveal that the incudo-malleolar joint is mobile rather than functionally fixed [49]. The mouse malleus exhibits a wide, thin, and flat transversal lamina not apparent in humans [47]. In mice, the processus anterior fuses to membranous bones, namely the goniale and the tympanic ring, while in humans the processus anterior is reduced to a small spicule of bone [41]. The stapes of mice and humans also differs: in mice, the anterior crus is curved and the posterior crus is more straight whereas in humans, the anterior crus is more straight than the posterior crus. It is worth noting that the malleus head relative to body size is massively enlarged in species such as the golden mole, demonstrating significant variability in allometric relationships of "the smallest" bones [48].
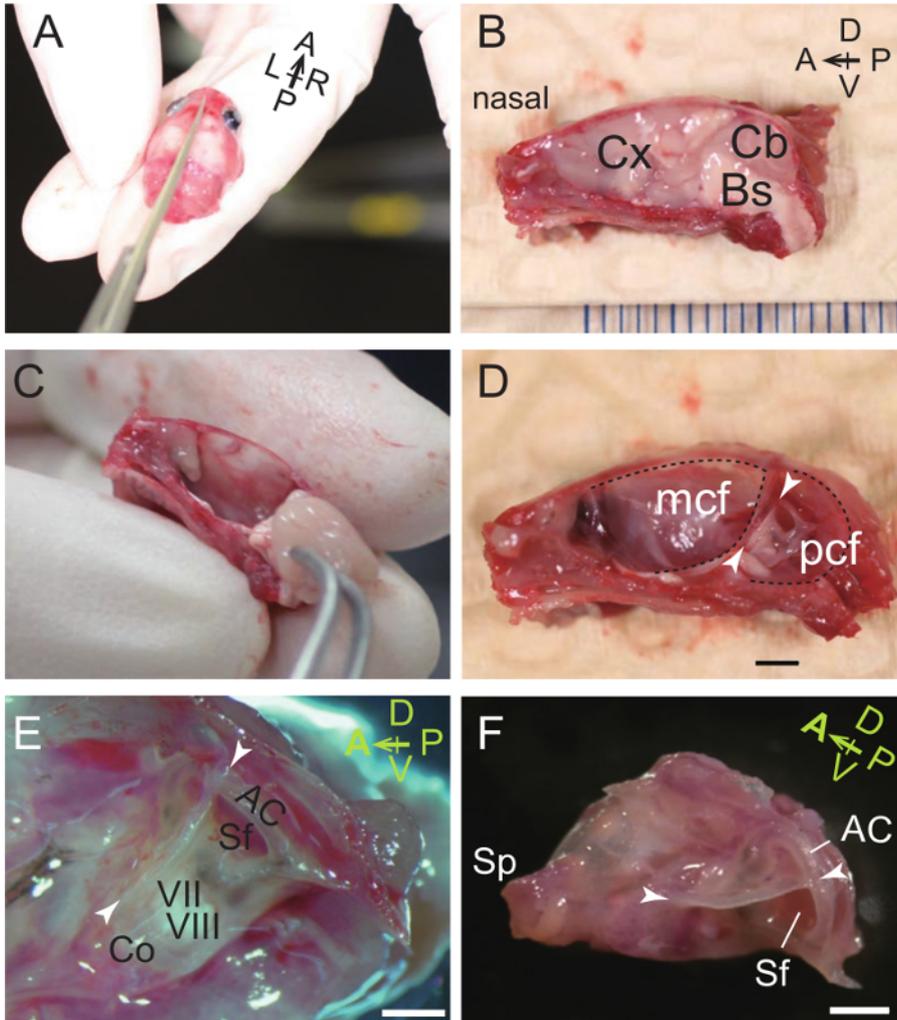
Figure 1. Dissection of the Auditory Bulla and Capsule. **(A)** The skull of a P31 mouse is split into right and left halves. A, anterior; P, posterior; L, left; R, right. **(B)** Medial surface of the right half of the bisected, skinned head. Cx, cerebral cortex; Cb, cerebellum; Bs, brainstem. D, dorsal; V, ventral. **(C)** Removal of brain with forceps. **(D)** Medial view of the auditory capsule in the right skull. The dorsal crest (arrowheads) lies between the middle cranial fossa (mcf) and posterior cranial fossa (pcf) and separates dorso-anterior and ventro-posterior surfaces of the auditory capsule. Scale bar, 2 mm. **(E)** Higher magnification of auditory bulla and capsule (medial view). Co, cochlea; VII, facial nerve; VIII, vestibulocochlear nerve; AC, anterior (superior) semicircular canal; Sf, subarcuate fossa, which houses the cerebellar paraflocculus. Scale bar, 1 mm. **(F)** Micrograph of isolated auditory bulla and capsule (medial view). Sp, styliform process. Scale bar, 1 mm. **(A - E)**, P31 mouse. **(F)**, P33 mouse. Please click here to view a larger version of this figure.
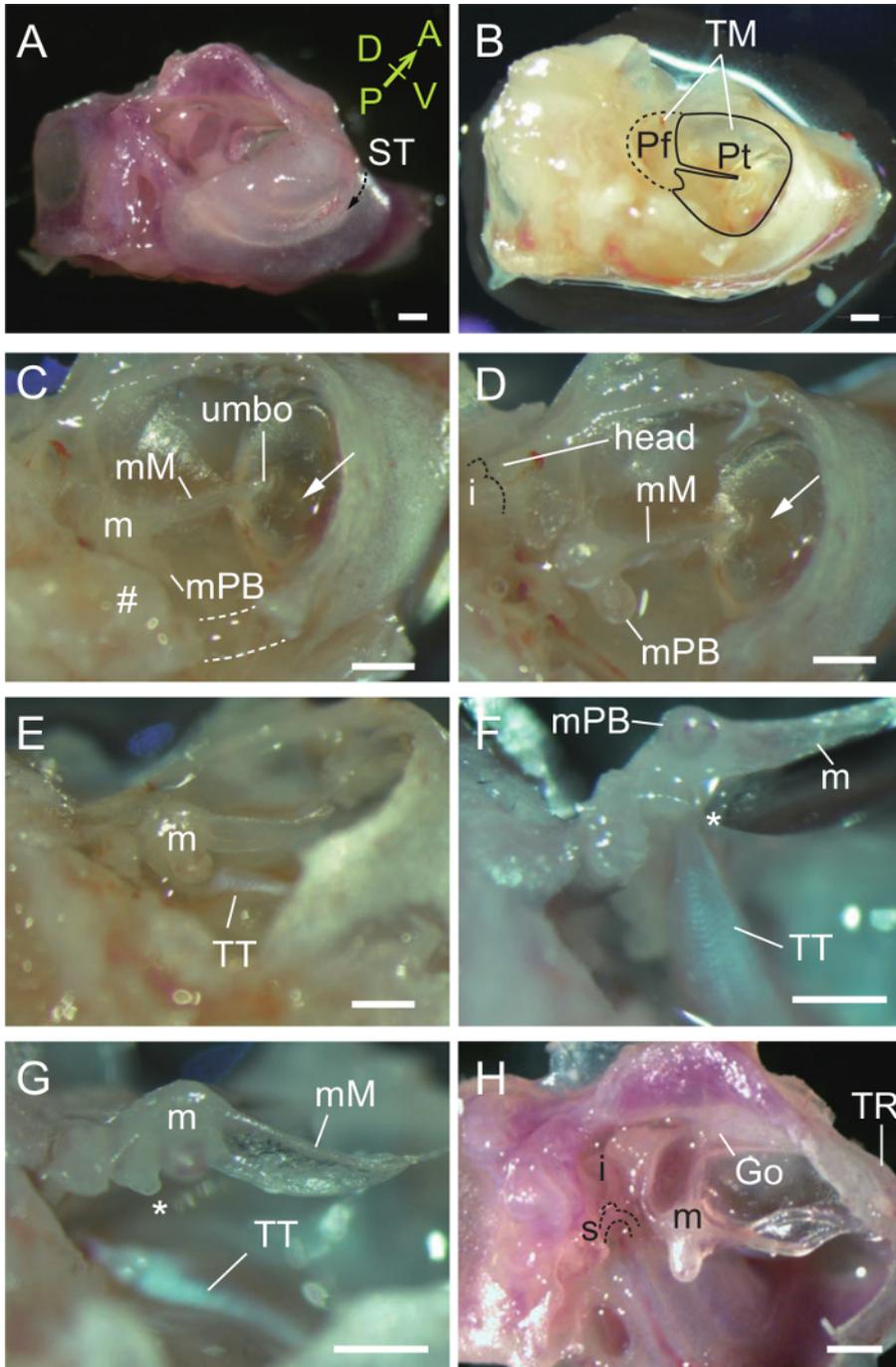
Figure 2. Dissection of the Malleus. (A) Ventrolateral view of a right auditory bulla and capsule. The sulcus tympanicus (ST, dashed arrow) is the attachment site of the tympanic membrane. The bone lateral to the ST is part of the external ear, and the bone medial to the ST forms the floor of the middle ear cavity. A, anterior; P, posterior; D, dorsal; V, ventral. **(B)** View after removal of the external auditory canal to reveal the tympanic membrane (TM) including the pars flaccida (Pf) and pars tensa (Pt). **(C)** Removal of parts of the tympanic bone (dotted lines and #) near the malleal processus brevis (mPB). m, malleus; mM, malleal manubrium. Arrow, air bubble in the middle ear cavity seen through the tympanic membrane. **(D)** Exposed malleus. Malleus head is indicated. Dotted line indicates the articular surface of the incus. **(E)** Tendon of the tensor tympani muscle (TT) attached to the malleus. **(F)** The tensor tympani is pulled when the malleus is lifted. *, muscular process. **(G)** Tensor tympani is cut using a needle. **(H)** Three auditory ossicles after removal of the tympanic membrane. The incudo-malleolar joint is dislocated. m, malleus; i, incus; s, stapes; Go, goniale (fused to the malleus and tympanic ring, TR). All scale bars, 0.5 mm. **(A, H)**, P33 mouse. **(B - G)**, P31 mouse. Please click here to view a larger version of this figure.
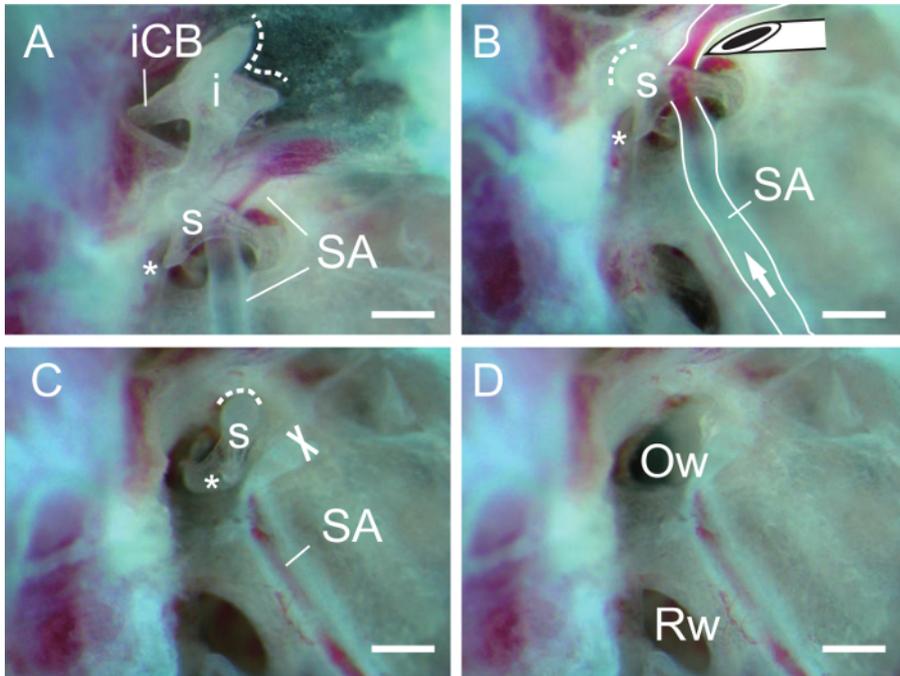
Figure 3. Dissection of the Incus and Stapes. (A) Incus and stapes after removal of the malleus. The stapedial artery (SA) passes through the stapes (s). Dotted line indicates the articular surface of the incus. Note that the short crus (iCB, crus breve) of the incus (i) is fixed by the posterior ligament (not shown). Asterisk, muscular process of the stapes. **(B)** Stapes after removal of the incus. Needle tip is used to cut the stapedial artery (SA). Arrow, direction of blood flow. Dotted line indicates articular surface of the stapes. **(C)** The stapedial artery is removed from the stapes. X indicates the cut end of the stapedial artery (SA). **(D)** The oval window (Ow, *fenestra ovalis* or *fenestra vestibuli*) is visible after removal of the stapes. Rw, round window (*fenestra rotunda* or *fenestra cochleae*). Scale bars, 0.5 mm. Please click here to view a larger version of this figure.
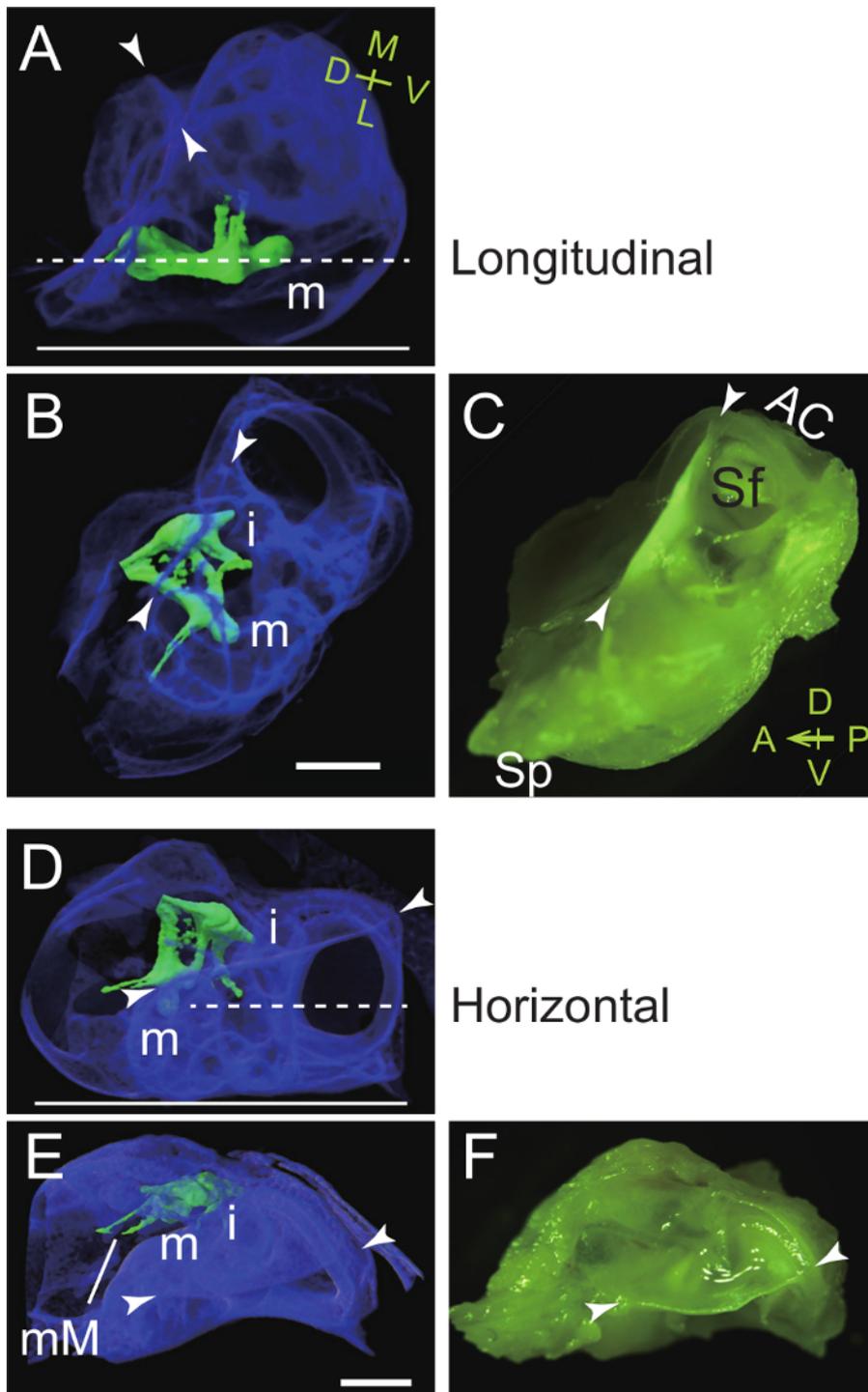
Figure 4. Orienting the Auditory Bulla and Capsule during Embedding for Longitudinal (parasagittal, A - C) and Horizontal (D - E) Sectioning of the Malleus. (A - C) The neck and transversal lamina of the malleus are placed parallel to the bottom of embedding dish. **(A)** Side view: micro-CT image to show embedding of the right malleus in the bulla (pseudocolored blue). The malleus and incus are pseudocolored green. Dashed line, the desired cutting plane. Solid line, bottom of embedding dish. m, malleus; arrowheads, dorsal crest. M, medial; L, lateral; D, dorsal; V, ventral. **(B)** Top view: Micro-CT image. Note that the anterior end of the bulla (styliform process) was removed. i, incus. **(C)** Top view: micrograph (taken with a color filter). AC, anterior (superior) semicircular canal; Sf, subarcuate fossa; Sp, styliform process. A, anterior; P, posterior; D, dorsal; V, ventral. **(D - F)** The processus brevis of the malleus is placed perpendicular to the bottom of embedding dish. **(D)** Side view: Micro-CT image to show embedding of the right malleus. Dashed line, the desired cutting plane. Solid line, bottom of embedding dish. **(E)** Top view: Micro-CT image. mM, malleal manubrium. **(F)** Top view: micrograph (taken with a color filter). Scale bars, 1 mm. Micro-CT images were obtained at a voxel resolution of 5 µm, as previously described [7]. Please click here to view a larger version of this figure.
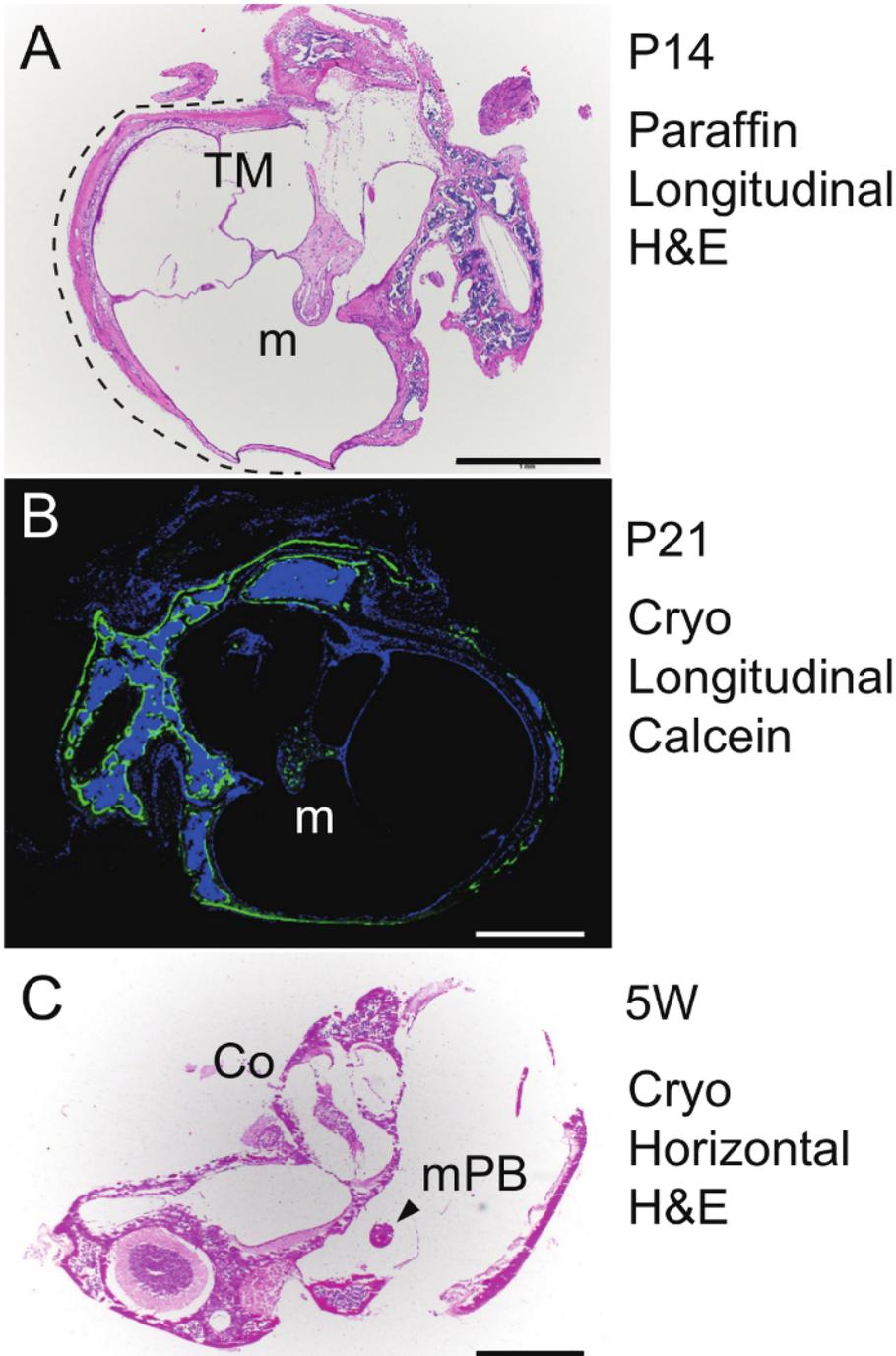
Figure 5. Histology. (A) H&E staining. Longitudinal (parasagittal) section of the paraffin-embedded right malleus (m) in the auditory bulla (dotted line) at P14. TM, tympanic membrane. **(B)** Calcein bone labeling. Longitudinal section of the frozen, undecalcified left malleus (m) in the auditory bulla at P21. Counterstain, DAPI. **(C)** H&E staining. Horizontal section of the frozen, undecalcified left malleal processus brevis (mPB) in the auditory bulla and capsule (5-week-old mouse). Co, cochlea. Scale bars, 1 mm. Please click here to view a larger version of this figure.
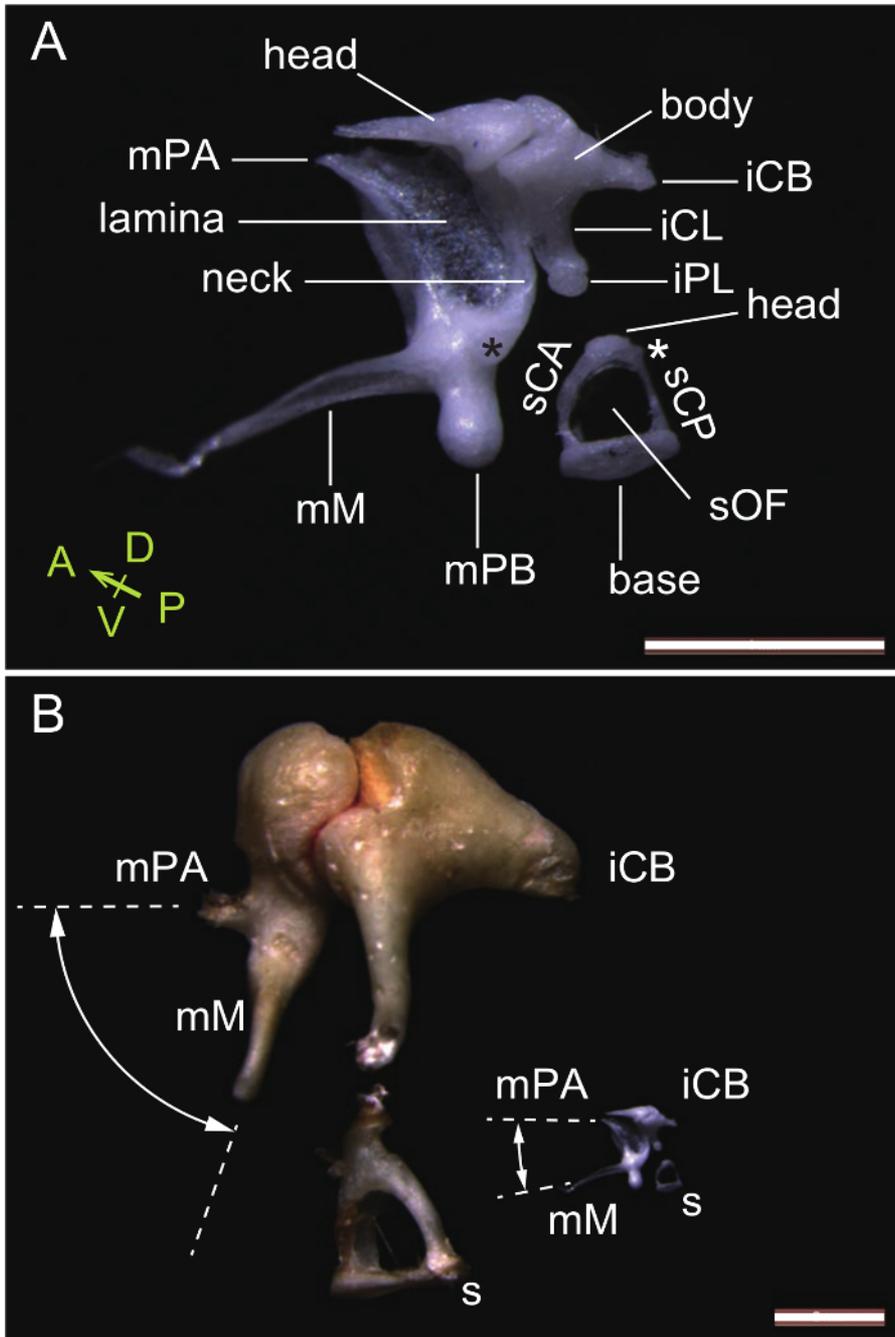
Figure 6. Medial View of Auditory Ossicles. (A) Right auditory ossicles of P31 mouse. A, anterior; P, posterior; D, dorsal; V, ventral. Scale bar, 1 mm. malleus head (Caput mallei, Capitulum mallei); neck (Collum mallei); lamina (transversal lamina); mM (Manubrium mallei); black asterisk (muscular process of the malleus); mPA (Processus anterior, Processus gracilis); mPB (processus brevis); incus body (Corpus incudis); iCB (Crus breve, short crus, short process); iCL (Crus longum, long crus, long process); iPL (Processus lenticularis, lenticular process, Sylvian apophysis); stapes head (Caput stapedis); white asterisk (muscular process of the stapes); sCA (Crus anterius, anterior crus); sCP (Crus posterius, posterior crus); base (Basis stapedis, footplate); sOF (obturator foramen, intercrural foramen). **(B)** Right auditory ossicles of a 76-year-old human female (Courtesy of Department of Anatomy, Keio University School of Medicine). The ossicles of P31 mouse (lower right) are imaged at the same magnification as that used for human ossicles. Curved arrows indicate the angle between the anatomical axis and the manubrium (dotted lines). Scale bar, 2 mm. Please click here to view a larger version of this figure.

Discussion

Here, we present a method useful to isolate the auditory bulla and capsule in postnatal mice. Prior to P12, tissues are fragile and can become damaged during isolation. After P12, the auditory bulla and capsule can be easily isolated from surrounding tissues. Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occur most actively from P6 onwards and are complete by P14[50]. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through

the cavitation process. The resultant air in the middle ear cavity can impede contact between tissues and liquids during fixation, decalcification and embedding. It is easier to remove air from the isolated auditory bulla by cutting off the anterior end (styliform process) rather than trying to do so in the unisolated bulla. Second, orientation of the malleus (and the tympanic membrane) is not vertical in the head. It is therefore easier to section the malleus in desired planes by embedding the isolated auditory bulla and capsule in a given orientation.

Once isolated, auditory bulla and capsules are useful for numerous analyses. For example, high resolution X-ray micro-CT can reveal bone microstructure morphology such as osteogenic capillaries in the malleus [14]. The stereofluorescence dissecting microscope is a powerful tool to visualize structures in evaluating reporter mice expressing fluorescent proteins in the middle or inner ear [33]. In addition, various *in vivo* or *ex vivo* fluorescence labeling methods and whole mount immunofluorescence detection could be undertaken. Light sheet fluorescence microscopy is also useful for three-dimensional analysis [51]. Although not described here, diverse anatomical structures associated with the auditory bulla and capsule such as peripheral nerves, blood vessels, and the tympanic membrane in the middle ear can also be evaluated using this protocol.

Note that paraffin sectioning requires decalcification of bone tissues before embedding and therefore does not allow analysis of mineralization. By contrast, the Kawamoto film method [43] used to prepare frozen sections can be performed without decalcification and is suitable for mineralization studies using *in vivo* bone-labeling techniques or special staining such as Alizarin staining. Cryo-sectioning conditions should be optimized according based on mouse age. For example, a less cool temperature inside the cryostat chamber is recommended for older mouse specimens to minimize damage to sections.

In mouse, the correct term for the prominent semi-spherical protrusion of the malleus is "orbicular apophysis". Nevertheless, the term "processus brevis" has been widely used to indicate the orbicular apophysis for more than two decades, particularly among mouse developmental biologists [16,20,22-25]. "Processus brevis" originally referred to the lateral process (processus lateralis), which differs from the orbicular apophysis. In humans, a lateral process resembling a slight conical projection forms the general line of attachment to the tympanic membrane, extending from the manubrium (not seen in **Figure 6B**, medial view). In mice, the lateral process is also a projection of the manubrium at the opposite end to the umbo [48]. The pars flaccida of the tympanic membrane is above the lateral process of the malleus. Orbicular apophysis is not apparent in the human malleus.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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