

# Journal of Visualized Experiments

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

<b>Manuscript Number:</b>	JoVE55054R1
<b>Full Title:</b>	Dissection of the Auditory Bulla in Postnatal Mice: Isolation of the Middle Ear Bones and Histological Analysis
<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Keywords:</b>	auditory ossicles, middle ear, inner ear, malleus, incus, stapes, tympanic bulla, tympanic membrane, tensor tympani, otic capsule, cochlea, stapedial artery
<b>Manuscript Classifications:</b>	1.2.835.232: Bone and Bones; 1.9: Sense Organs; 1.9.246: Ear; 1.9.246.397: Ear, Middle; 1.9.246.397.247: Ear Ossicles
<b>Corresponding Author:</b>	Koichi Matsuo Keio University School of Medicine Tokyo, JAPAN
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	kmatsuo@keio.jp
<b>Corresponding Author's Institution:</b>	Keio University School of Medicine
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Ayako Sakamoto
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Ayako Sakamoto Yukiko Kuroda Sho Kanzaki
<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	<p>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 three-dimensional 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 to prepare 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.</p>
<b>Author Comments:</b>	<p>We would like to submit Movies 1, 2, and 3, which show our procedures under microscope. We believe that these are helpful and we hope that you will include them in the final publication. Please let us know if any revisions are necessary with the movies. Here are some explanations on the movies.</p> <p>Movies 1-3 for procedures under microscope.</p>

	<p>Movie 1: Isolation of the auditory bulla and capsule ( 0 ' 59")  Protocol 1.9  Pink-colored circle indicates the auditory bulla (middle ear and a part of outer ear) and green triangle indicates the auditory capsule (inner ear).  The orientation of the bulla and capsule is shown.  Occipital bone (basooccipital and exoccipital) is loosened and cut away.  Parietal and interparietal bone is also loosened and cut away.</p> <p>Movie 2: Isolation of Auditory Ossicles  Movie 2a: Isolation of the malleus (1'06")  Movie 2b: Isolation of the incus and stapes (1'04")  Protocol 2.1.1 - 2.2.2  These steps are usually performed in PBS, but to avoid reflection, movie was taken in the absence of PBS.</p> <p>Movie 3: Preparation for embedding (1'02")  Cut the anterior end of the bulla  Protocol 3.1.2, 3.2.2  Removal air bubbles of the bulla  Protocol 3.2.4  Air bubble in the middle ear was aspirated with a needle (and syringe).  Air bubble in the outer ear was aspirated with a needle.</p>
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

**TITLE:**

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

**AUTHORS:**

Sakamoto, Ayako  
Laboratory of Cell and Tissue Biology  
Keio University School of Medicine  
Tokyo, Japan  
ayasaka@keio.jp

Kuroda, Yukiko  
Laboratory of Cell and Tissue Biology  
Keio University School of Medicine  
Tokyo, Japan  
kuro.z6@keio.jp

Kanzaki, Sho  
Department of Otolaryngology Head and Neck Surgery  
Keio University School of Medicine  
Tokyo, Japan  
skan@keio.jp

Matsuo, Koichi  
Laboratory of Cell and Tissue Biology  
Keio University School of Medicine  
Tokyo, Japan  
kmatsuo@keio.jp

**CORRESPONDING AUTHOR:**

Matsuo, Koichi, M.D., Ph.D.  
+81-3-5843-6203

**KEYWORDS:**

auditory ossicles, middle ear, inner ear, malleus, incus, stapes, tympanic bulla, tympanic membrane, tensor tympani, otic capsule, cochlea, stapedial artery

**SHORT ABSTRACT:**

We present a protocol to isolate the auditory bulla, capsule, and ossicles from postnatal mice for whole mount and histological analysis.

**LONG 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 three-dimensional 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 to prepare 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.

## 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<sup>1,2</sup>. Hearing function can be evaluated in mice by measuring auditory brain stem response (ABR) thresholds<sup>3-6</sup>, and vibration of the malleus behind the tympanic membrane can be monitored using laser Doppler vibrometry (LDV)<sup>7</sup>. By combining ABR, LDV, and distortion product otoacoustic emission (DPOAE) measurements, conductive hearing loss can be discriminated from sensorineural impairment<sup>8</sup>.

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<sup>9</sup>. Mouse models of otitis media could prove useful in understanding the pathogenesis and in developing treatments<sup>10,11</sup>.

Murine ossicles, which (except for the goniale part of the malleus) are formed by endochondral ossification<sup>12,13</sup>, 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<sup>14</sup>. Second, aberrant bone metabolism, such as excessive or deficient bone resorption, or impaired interactions among bone cells<sup>15</sup>, can be analyzed as a potential contributor to hearing loss<sup>3,4,7</sup>. Third, abnormal ossicle morphogenesis is reported in several gene-deficient mice, such as animals lacking *Hoxa2*<sup>16-19</sup>, *Msx1*<sup>20-22</sup>, *Prrx1*<sup>23</sup>, *Gooseoid (Gsc)*<sup>24,25</sup>, *Bapx1*<sup>13</sup>, *Tshz1*<sup>26</sup>, *Dusp6 (Mkp3)*<sup>27</sup>, *Noggin (Nog)*<sup>28</sup>, *Fgfr1*<sup>29</sup>, thyroid hormone receptors (*Thra*, *Thrb*)<sup>5</sup>, *Bcl2*<sup>30</sup> and others<sup>1,31</sup>, or in mice overexpressing *Hoxa2*<sup>32</sup>. Finally, despite their small size, structures associated with ossicles such as muscles<sup>33</sup> and joints<sup>34,35</sup> 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 stapedia artery, which passes through the ring of the stapes, persists throughout life<sup>36</sup>, whereas in humans, the embryonic stapedia 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<sup>37</sup>. 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<sup>38-40</sup>.

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 (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**

1.1. Euthanize mice in a jar containing a platform above paper towels soaked in isoflurane or sevoflurane until respiratory ventilation ceases for more than a minute and then perform cervical dislocation. Be careful to avoid direct contact of mice with the soaked paper towels.

1.2. 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.

1.3. Decapitate mice at the cervical region using 14-cm sharp surgical scissors.

1.4. Peel skin completely towards the nose. Cut off all skin together with the snout and incisors.

1.5. Insert scissors into the mouth and cut masseter muscles on both sides.

1.6. Open the jaw carefully and remove the tongue and lower jaw together.

1.7. Using sharp scissors, split skull and skull base into two halves along the midsagittal plane (Figure 1AB).

1.8. 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 1CD).

1.9. Dissect out the bulla and capsule with the surrounding skull bone (Figure 1E).

1.10. Transfer the specimen to a dish containing phosphate-buffered saline (PBS) pH 7.4 at

room temperature.

1.11. 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, Movie 1). 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 styloid process (Sp), which supports the tympanic opening of the Eustachian tube<sup>41</sup>, is distinct from the styloid process of the temporal bone.

## **2. Isolation of Auditory Ossicles: Malleus, Incus and Stapes (Movie 2)**

### **2.1. Malleus**

2.1.1. 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 2AB).

2.1.2. 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 2DE).

2.1.3. Lift the malleus (Figure 2F) and cut the tensor tympani muscle with the beveled edge of a 27G-needle (Figure 2G). Note that the malleal manubrium firmly attaches to the tympanic membrane, as is seen in other mammals.

2.1.4. Detach the tympanic membrane carefully from the manubrium, which is fragile. Remove the tympanic bone to reveal the three auditory ossicles.

2.1.5. Dislocate the malleus from the incus at the ossicular joint (Figure 2H).

2.1.6. Isolate the malleus by fracturing the anterior process at the goniale.

### **2.2. Incus and Stapes**

2.2.1. Isolate the incus by cutting off the posterior ligament of the incus at the short crus (Figure 3A).

2.2.2. Isolate the stapes by cutting off the stapedia artery near the stapes with the beveled edge of a 27G-needle (Figure 3BC). If necessary, cut the tendon of the stapedia muscle at the muscular process of the stapes with the needle.

2.2.3. 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**

### **3.1. Preparation for embedding in paraffin blocks**

3.1.1. Isolate the bulla and capsule as described in Section 1.

3.1.2. 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 overnight on a tube rotator.

Caution: PFA is toxic and should be handled carefully.

3.1.3. Wash once with PBS.

3.1.4. 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, in a 2 mL tube. Change the buffer every other day.

3.1.5. 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).

3.1.6. On a tissue processor, dehydrate specimens in a graded series of ethanol solutions (70%, two changes of 95%, three changes of 100%, each 1 hr), clear in xylene (four changes, each 1 hr at 40°C), and infiltrate specimens with molten paraffin wax<sup>42</sup>. Optionally, substitute xylene with commercial tissue clearing solution (e.g., Histo-Clear).

3.1.7. Unload specimens from the processor, and remove them from their cassettes.

3.1.8. On a tissue embedding console system, place specimens into molds filled with molten paraffin wax. Proceed to embedding (Section 4).

### **3.2. Preparation for embedding in frozen blocks (Kawamoto's film method) <sup>43</sup>**

3.2.1. Isolate the bulla and capsule as described in Section 1.

3.2.2. Cut the anterior end of the bulla (the styliform process) off with scissors (Movie 3), 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 overnight on a tube rotator.

3.2.3. Wash bulla and capsule quickly in PBS and immediately immerse in liquid cryo-embedding compound at 4 °C.

3.2.4. 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 (Movie 3). 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.

#### **4.1. Longitudinal (parasagittal) sectioning of the malleus**

4.1.1. 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 degrees to the vertical in the mouse head (Figure 4A; Figure 59 in Kampen<sup>44</sup>).

#### **4.2. Horizontal sectioning of the malleus**

4.2.1. 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).

#### **4.3. Frontal sectioning (cross sections) of manubrium and tympanic membrane<sup>5</sup>**

4.3.1. Place the malleal manubrium in warm paraffin (or cryo-embedding compound) such that it is perpendicular to the bottom of the embedding dish.

4.4. 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).

4.5. 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)<sup>3</sup>, or by immunohistochemistry. Undecalcified cryosections are suitable for bone labeling using fluorochromes<sup>14</sup>, alizarin red staining for calcium, and immunofluorescence<sup>42</sup>.

### **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 styloid process at the anterior end of the bulla, the dorsal crest, anterior semicircular canal, and the subarcuate fossa (Figure 1F). Micro-computed 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 sectioning of the malleus, auditory bullae were isolated from a postnatal day 14 (P14) mouse. 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). For bone labeling, 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<sup>43</sup>. 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  $\mu$ m 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<sup>45</sup>) 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<sup>6,46-48</sup>. In human ossicles, vibrometric studies reveal that the incudo-malleolar joint is mobile rather than functionally fixed<sup>49</sup>. The mouse malleus exhibits a wide, thin, and flat transversal lamina not apparent in humans<sup>47</sup>. 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<sup>41</sup>. 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<sup>48</sup>.

### 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, brain stem. 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.

### 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 and H), P33 mouse. (B-G), P31 mouse.

### Figure 3. Dissection of the incus and stapes.

**(A)** Incus and stapes after removal of the malleus. The stapedia 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 stapedia artery (SA). Arrow, direction of blood flow. Dotted line indicates articular surface of the stapes. **(C)** The stapedia artery is removed from the stapes. X indicates the cut end of the stapedia 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.

### Figure 4. Orienting the auditory bulla and capsule during embedding for longitudinal (parasagittal, A-C) and horizontal sectioning (D-E) 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 (styliiform process) was removed. i, incus. **(C)** Top view: micrograph (taken with a color filter). AC, anterior (superior) semicircular canal; Sf, subarcuate fossa; Sp, styliiform 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  $\mu\text{m}$ , as previously described <sup>7</sup>.

### 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.

**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 anterior, 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 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.

**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<sup>50</sup>. 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 (styliiform 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<sup>14</sup>. The stereofluorescence dissecting microscope is a powerful tool to visualize structures in evaluating reporter mice expressing fluorescent proteins in the middle or inner ear<sup>33</sup>. 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<sup>51</sup>. 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 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<sup>16,20,22-25</sup>. “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 Fig. 6B, medial view). In mice, the lateral process is also a projection of the manubrium at the opposite end to the umbo<sup>48</sup>. The pars flaccida of the tympanic membrane is above the lateral process of the malleus. Orbicular apophysis is not apparent in the human malleus.

#### ACKNOWLEDGMENTS:

The authors thank Masaki Yoda and Elise Lamar for critical reading of the manuscript, Kazumasa Takenouchi for help with histology, Mari Fujiwara for help with microscopy and Makoto Morikawa for help in photographing human and mouse auditory ossicles.

#### DISCLOSURES:

The authors have nothing to disclose.

#### REFERENCES

- 1 Mallo, M. Formation of the middle ear: recent progress on the developmental and molecular mechanisms. *Dev Biol* **231**, 410-419, doi:10.1006/dbio.2001.0154 (2001).
- 2 Manley, G. A. An evolutionary perspective on middle ears. *Hear Res* **263**, 3-8, doi:10.1016/j.heares.2009.09.004 (2010).
- 3 Kanzaki, S., Ito, M., Takada, Y., Ogawa, K. & Matsuo, K. Resorption of auditory ossicles and hearing loss in mice lacking osteoprotegerin. *Bone* **39**, 414-419 (2006).
- 4 Kanzaki, S., Takada, Y., Ogawa, K. & Matsuo, K. Bisphosphonate therapy ameliorates hearing loss in mice lacking osteoprotegerin. *J Bone Miner Res* **24**, 43-49, doi:10.1359/jbmr.080812 (2009).
- 5 Cordas, E. A. *et al.* Thyroid hormone receptors control developmental maturation of the middle ear and the size of the ossicular bones. *Endocrinology* **153**, 1548-1560, doi:10.1210/en.2011-1834 (2012).
- 6 Dong, W., Varavva, P. & Olson, E. S. Sound transmission along the ossicular chain in common wild-type laboratory mice. *Hear Res* **301**, 27-34, doi:10.1016/j.heares.2012.11.015 (2013).
- 7 Kanzaki, S. *et al.* Impaired vibration of auditory ossicles in osteopetrotic mice. *Am J Pathol* **178**, 1270-1278, doi:10.1016/j.ajpath.2010.11.063 (2011).
- 8 Qin, Z., Wood, M. & Rosowski, J. J. Measurement of conductive hearing loss in mice. *Hear Res*, doi:10.1016/j.heares.2009.10.002 (2009).
- 9 Klein, J. O. Is acute otitis media a treatable disease? *N Engl J Med* **364**, 168-169, doi:10.1056/NEJMe1009121 (2011).
- 10 Rosch, J. W. *et al.* A live-attenuated pneumococcal vaccine elicits CD4<sup>+</sup> T-cell dependent class switching and provides serotype independent protection against acute otitis

media. *EMBO Mol Med* **6**, 141-154, doi:10.1002/emmm.201202150 (2014).

11 Li, X. *et al.* Otitis media in sperm-associated antigen 6 (*Spag6*)-deficient mice. *PLoS One* **9**, e112879, doi:10.1371/journal.pone.0112879 (2014).

12 Rodríguez Vázquez, J. F., Mérida Velasco, J. R. & Jiménez Collado, J. A study of the os goniale in man. *Acta Anat (Basel)* **142**, 188-192 (1991).

13 Tucker, A. S., Watson, R. P., Lettice, L. A., Yamada, G. & Hill, R. E. *Bapx1* regulates patterning in the middle ear: altered regulatory role in the transition from the proximal jaw during vertebrate evolution. *Development* **131**, 1235-1245, doi:10.1242/dev.01017 (2004).

14 Matsuo, K. *et al.* Osteogenic capillaries orchestrate growth plate-independent ossification of the malleus. *Development* **142**, 3912-3920, doi:10.1242/dev.123885 (2015).

15 Matsuo, K. Cross-talk among bone cells. *Curr Opin Nephrol Hypertens* **18**, 292-297, doi:10.1097/MNH.0b013e32832b75f1 (2009).

16 Rijli, F. M. *et al.* A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. *Cell* **75**, 1333-1349 (1993).

17 Mallo, M. & Gridley, T. Development of the mammalian ear: coordinate regulation of formation of the tympanic ring and the external acoustic meatus. *Development* **122**, 173-179 (1996).

18 O'Gorman, S. Second branchial arch lineages of the middle ear of wild-type and *Hoxa2* mutant mice. *Dev Dyn* **234**, 124-131, doi:10.1002/dvdy.20402 (2005).

19 Santagati, F., Minoux, M., Ren, S. Y. & Rijli, F. M. Temporal requirement of *Hoxa2* in cranial neural crest skeletal morphogenesis. *Development* **132**, 4927-4936, doi:10.1242/dev.02078 (2005).

20 Satokata, I. & Maas, R. *Msx1* deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* **6**, 348-356, doi:10.1038/ng0494-348 (1994).

21 Zhang, Z. *et al.* Malleal processus brevis is dispensable for normal hearing in mice. *Dev Dyn* **227**, 69-77 (2003).

22 Houzelstein, D., Cohen, A., Buckingham, M. E. & Robert, B. Insertional mutation of the mouse *Msx1* homeobox gene by an *nlacZ* reporter gene. *Mech Dev* **65**, 123-133 (1997).

23 Martin, J. F., Bradley, A. & Olson, E. N. The *paired*-like homeo box gene *MHox* is required for early events of skeletogenesis in multiple lineages. *Genes Dev* **9**, 1237-1249 (1995).

24 Yamada, G. *et al.* Targeted mutation of the murine *gooseoid* gene results in craniofacial defects and neonatal death. *Development* **121**, 2917-2922 (1995).

25 Rivera-Pérez, J. A., Mallo, M., Gendron-Maguire, M., Gridley, T. & Behringer, R. R. *gooseoid* is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* **121**, 3005-3012 (1995).

26 Coré, N. *et al.* *Tshz1* is required for axial skeleton, soft palate and middle ear development in mice. *Dev Biol* **308**, 407-420, doi:10.1016/j.ydbio.2007.05.038 (2007).

27 Li, C., Scott, D. A., Hatch, E., Tian, X. & Mansour, S. L. *Dusp6* (*Mkp3*) is a negative feedback regulator of FGF-stimulated ERK signaling during mouse development. *Development* **134**, 167-176, doi:10.1242/dev.02701 (2007).

28 Hwang, C. H. & Wu, D. K. *Noggin* heterozygous mice: an animal model for congenital conductive hearing loss in humans. *Hum Mol Genet* **17**, 844-853, doi:10.1093/hmg/ddm356 (2008).

29 Calvert, J. A. *et al.* A missense mutation in *Fgfr1* causes ear and skull defects in hush puppy mice. *Mamm Genome* **22**, 290-305, doi:10.1007/s00335-011-9324-8 (2011).

30 Carpinelli, M. R. *et al.* Anti-apoptotic gene *Bcl2* is required for stapes development and

hearing. *Cell death dis* **3**, e362, doi:10.1038/cddis.2012.100 (2012).

31 Chapman, S. C. Can you hear me now? Understanding vertebrate middle ear development. *Front Biosci (Landmark Ed)* **16**, 1675-1692 (2011).

32 Kitazawa, T. *et al.* Distinct effects of *Hoxa2* overexpression in cranial neural crest populations reveal that the mammalian hyomandibular-ceratohyal boundary maps within the styloid process. *Dev Biol* **402**, 162-174, doi:10.1016/j.ydbio.2015.04.007 (2015).

33 Wang, L. *et al.* *Scleraxis* is required for differentiation of the stapedius and tensor tympani tendons of the middle ear. *J Assoc Res Otolaryngol* **12**, 407-421, doi:10.1007/s10162-011-0264-5 (2011).

34 Amin, S. & Tucker, A. S. Joint formation in the middle ear: lessons from the mouse and guinea pig. *Dev Dyn* **235**, 1326-1333, doi:10.1002/dvdy.20666 (2006).

35 Amin, S., Matalova, E., Simpson, C., Yoshida, H. & Tucker, A. S. Incudomalleal joint formation: the roles of apoptosis, migration and downregulation. *BMC Dev Biol* **7**, 134, doi:10.1186/1471-213X-7-134 (2007).

36 Hiruma, T., Nakajima, Y. & Nakamura, H. Development of pharyngeal arch arteries in early mouse embryo. *Journal of anatomy* **201**, 15-29 (2002).

37 Treuting, P. M. & Dintzis, S. M. Special senses: ear. in *Comparative Anatomy and Histology: A Mouse and Human Atlas*. (eds Treuting, P. M. & Dintzis, S. M.) Ch. 22, 419-432 (Academic Press, 2012).

38 Mallo, M., Schrewe, H., Martin, J. F., Olson, E. N. & Ohnemus, S. Assembling a functional tympanic membrane: signals from the external acoustic meatus coordinate development of the malleal manubrium. *Development* **127**, 4127-4136 (2000).

39 Anthwal, N., Joshi, L. & Tucker, A. S. Evolution of the mammalian middle ear and jaw: adaptations and novel structures. *Journal of anatomy* **222**, 147-160, doi:10.1111/j.1469-7580.2012.01526.x (2013).

40 Takechi, M. & Kuratani, S. History of studies on mammalian middle ear evolution: a comparative morphological and developmental biology perspective. *J Exp Zool B Mol Dev Evol* **314**, 417-433, doi:10.1002/jez.b.21347 (2010).

41 Henson Jr, O. W. Comparative Anatomy of the Middle Ear. in *Auditory System. Anatomy, Physiology (Ear)* Vol. 1 *Handbook of Sensory Physiology* (ed W. D. Keidel, Neff, W. D.) Ch. 3, 39-110 (Springer Berlin Heidelberg, 1974).

42 *Handbook of Histology Methods for Bone and Cartilage*. (Humana Press, 2003).

43 Kawamoto, T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch Histol Cytol* **66**, 123-143 (2003).

44 Kampen, P. N. v. in *Gegenbaurs Morphologisches Jahrbuch* Vol. 34 321-722 (W. Engelmann, 1905).

45 Lee, J. H., Park, K., Kang, T. C. & Choung, Y. H. Three-dimensional anatomy of the temporal bone in normal mice. *Anat Histol Embryol* **38**, 311-315, doi:10.1111/j.1439-0264.2009.00946.x (2009).

46 Fleischer, G. Evolutionary principles of the mammalian middle ear. *Adv Anat Embryol Cell Biol* **55**, 3-70 (1978).

47 Lavender, D., Taraskin, S. N. & Mason, M. J. Mass distribution and rotational inertia of "microtype" and "freely mobile" middle ear ossicles in rodents. *Hear Res* **282**, 97-107, doi:10.1016/j.heares.2011.09.003 (2011).

48 Mason, M. J. Of mice, moles and guinea pigs: functional morphology of the middle ear in living mammals. *Hear Res* **301**, 4-18, doi:10.1016/j.heares.2012.10.004 (2013).

- 49 Willi, U. B., Ferrazzini, M. A. & Huber, A. M. The incudo-malleolar joint and sound transmission losses. *Hear Res* **174**, 32-44 (2002).
- 50 Richter, C. A. *et al.* Defects in middle ear cavitation cause conductive hearing loss in the *Tcof1* mutant mouse. *Hum Mol Genet* **19**, 1551-1560, doi:10.1093/hmg/ddq028 (2010).
- 51 Buytaert, J. A., Johnson, S. B., Dierick, M., Salih, W. H. & Santi, P. A. MicroCT versus sTSLIM 3D imaging of the mouse cochlea. *J Histochem Cytochem* **61**, 382-395, doi:10.1369/0022155413478613 (2013).

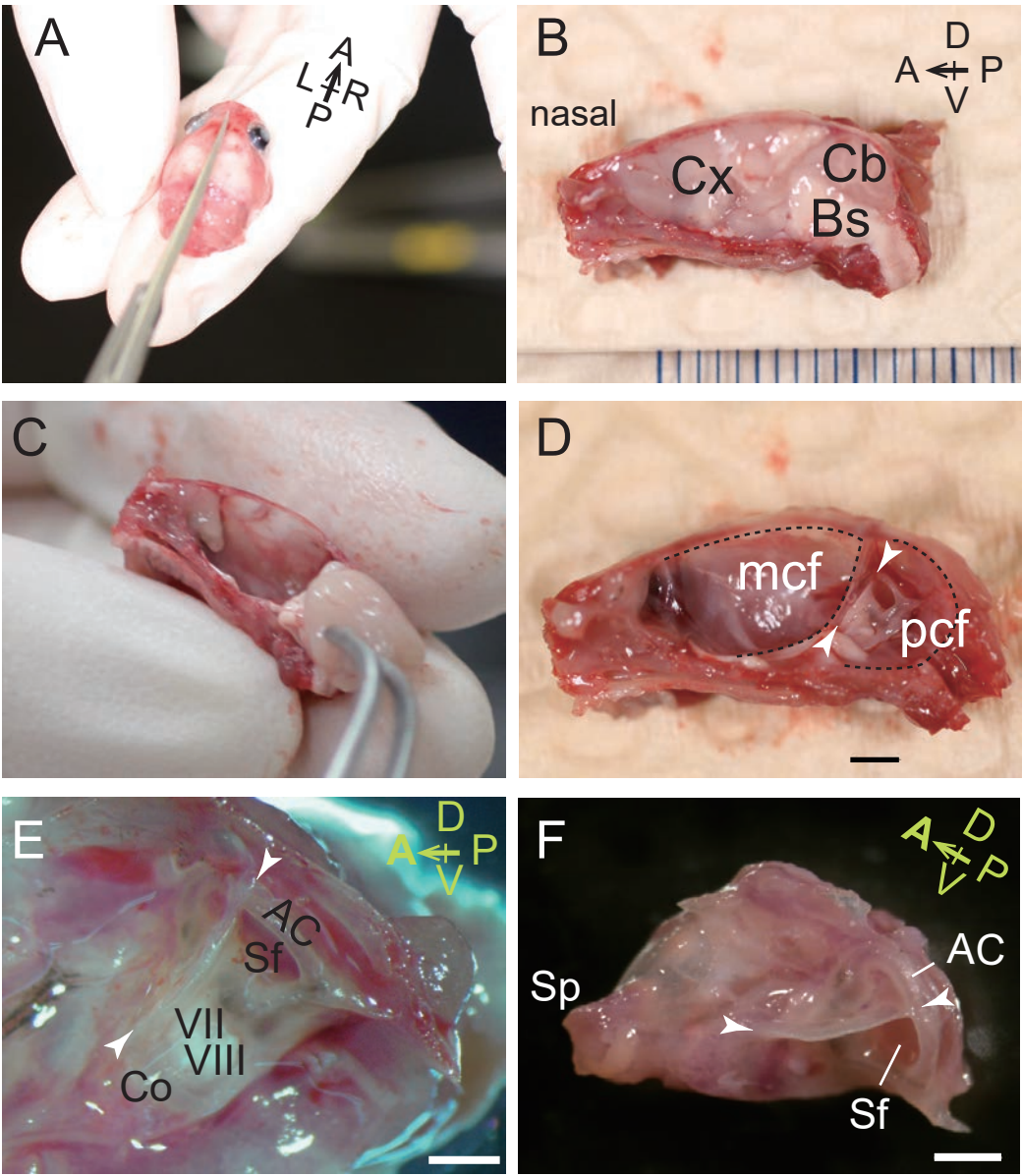


Fig. 1 Sakamoto *et al.*



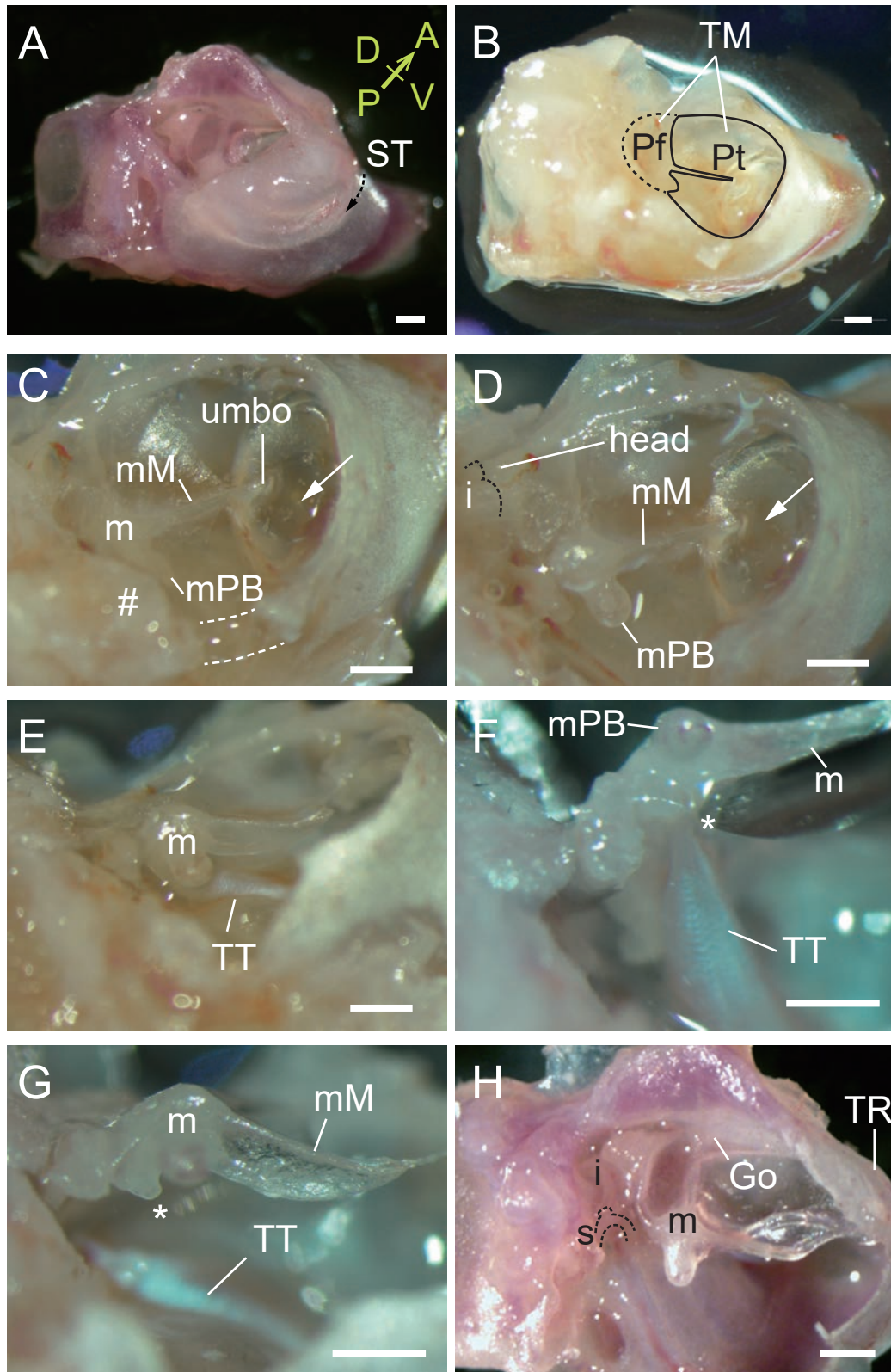


Fig. 2 Sakamoto *et al.*

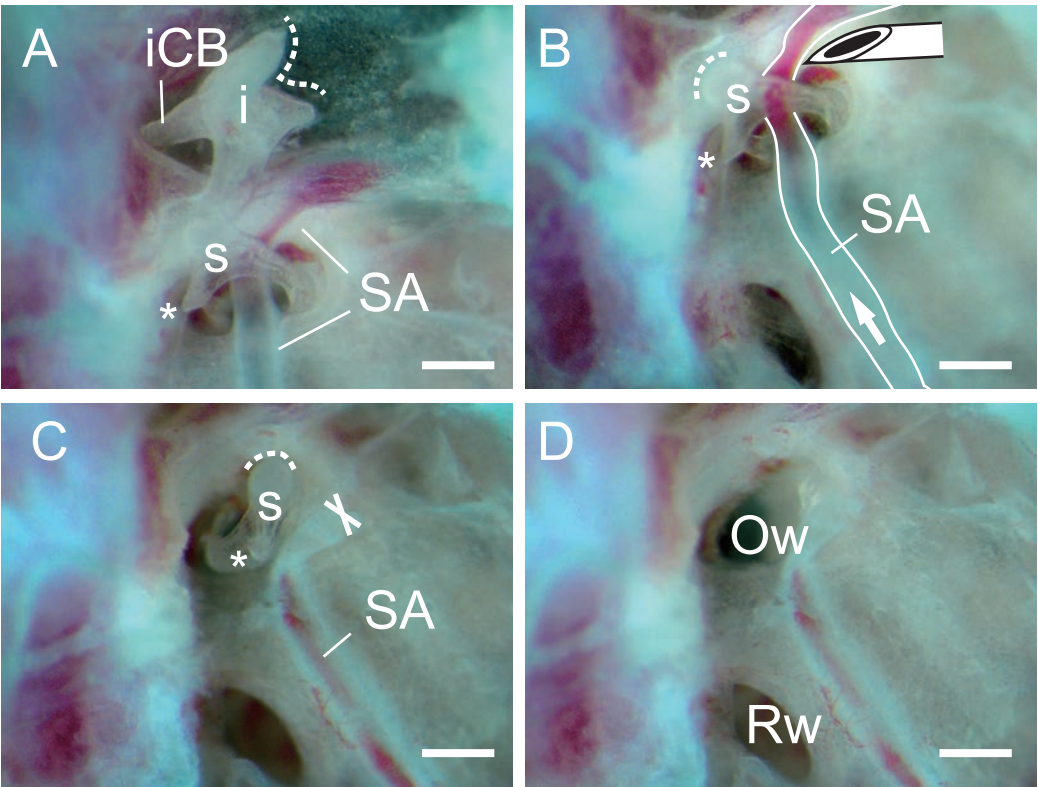


Fig. 3 Sakamoto *et al.*

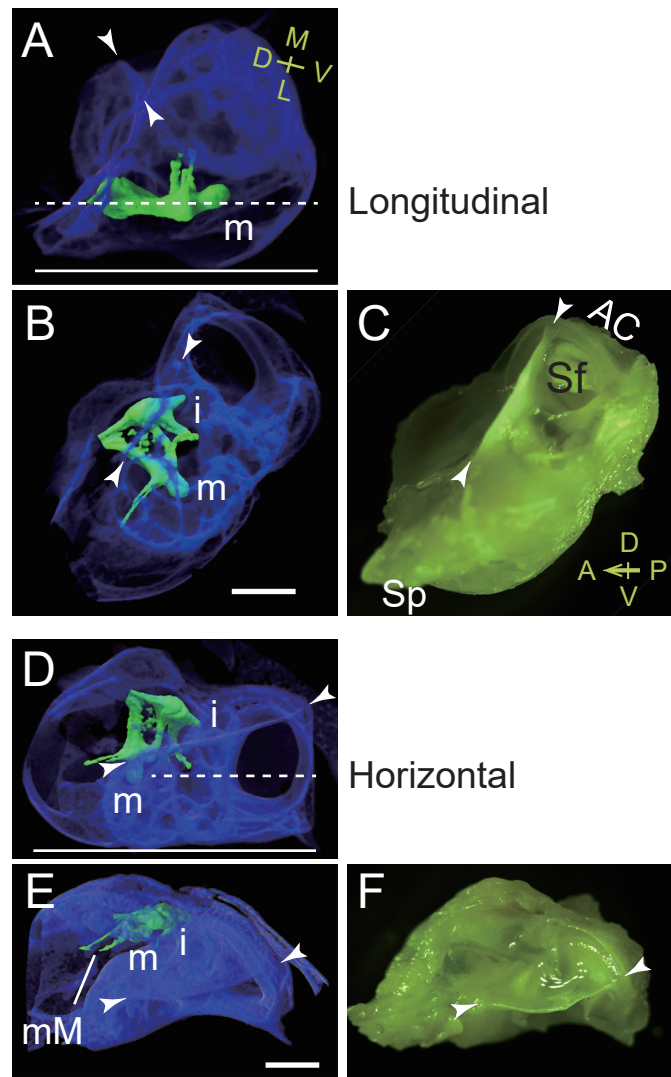


Fig. 4 Sakamoto *et al.*

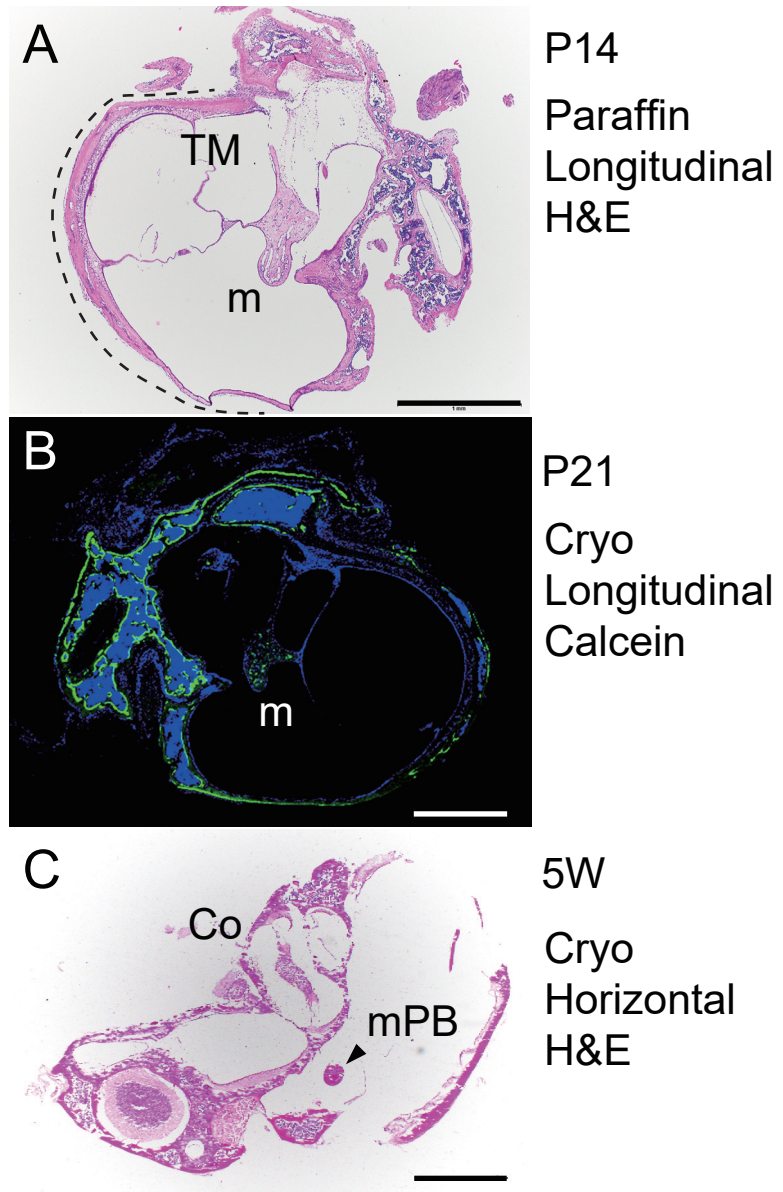


Fig. 5 Sakamoto *et al.*

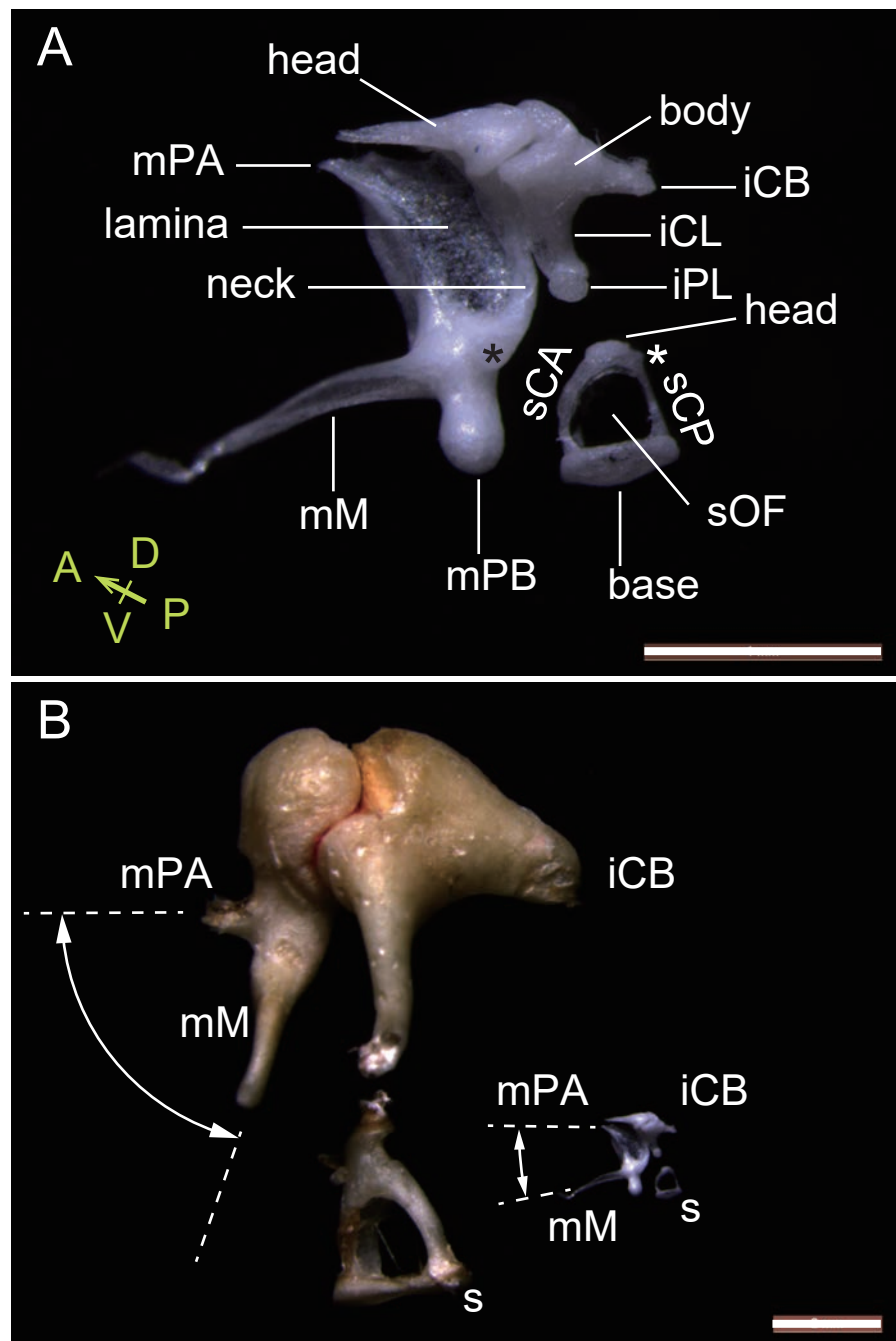


Fig. 6 Sakamoto *et al.*



Name Tools/Equipment	Company	Catalog Number
Paper towel	DAIO PAPER CORPORATION	703347
Glass Jar	Various	
14cm surgical scissors	Fine Science Tools (F.S.T.)	91400-14
Extra fine scissors-straight	Fine Science Tools (F.S.T.)	14084-08
Fine Forceps Angled 45°	Fine Science Tools (F.S.T.)	11063-07
Dissecting microscope	Nikon	SMZ800N
Dissecting microscope	Nikon	SMZ18
Injection needle 27G	TERUMO	NN-2719S
Syringe (1ml)	TERUMO	SS-01T
Marking Pin	Various	
Tube rotator RT-50	TAITEC	0000165-000
Cryostat	Leica	CM3050S
TC-65 Tungsten blade	Leica	14021626379
Stainless containers	Leica	
Cryofilm type IIC	Leica	
Silane coated slide (New Silane II)	Muto Pure Chemicals	511617
Cover glass	Matsunami	
Tissue processor	Sakura Finetek	VIP-5
Tissue Embedding Console System	Sakura Finetek	Tissue-Tek TEC 5
Sliding microtome for paraffin	Yamato Kohki Industrial	REM-710
Path Blade+pro for hard tissue	Matsunami	PB3503C
Micro-CT	RIGAKU	R_mCT2
Fluorescence microscope	KEYENCE	BZ-9000
<b>Reagents</b>		
Isoflurane	Maruishi pharmaceutical Co. Ltd	
NaCl	wako	191-01665
KCl	wako	285-14
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O	wako	196-02835
KH <sub>2</sub> PO <sub>4</sub>	wako	287-21
Paraformaldehyde(EM Grade)	TAAB	P001
EDTA-2Na	wako	15111-45
Trizma base	Sigma	T1503-1KG
Super Cryoembedding Medium	Leica	
Dry Ice	Various	
Hexane	wako	080-03423
Super Cryomounting Medium type R2	Leica	
Paraffin	Sakura Finetek	781001A0107
Histo-Clear	NDS	HS-200
Calcein	DOJINDO	340-00433
Hematoxylin	wako	131-09665
Eosin	wako	051-06515

## Comments

can be purchased from other vendors  
can be purchased from other vendors  
can be purchased from other vendors  
can be purchased from other vendors  
can be purchased from other vendors  
for routine dissection  
for movies

can be purchased from other vendors  
<http://www.leicabiosystems.com/histology-equipment/cryostats/details/product/leica-cm3050-s/>  
for Kawamoto's firm method  
for Kawamoto's firm method  
for Kawamoto's firm method  
can be purchased from other vendors  
can be purchased from other vendors  
can be purchased from other vendors  
can be purchased from other vendors  
can be purchased from other vendors  
for paraffin section  
<http://www.rigaku.com/en>

for PBS  
for PBS  
for PBS  
for PBS

for Kawamoto's firm method  
for Kawamoto's firm method  
for Kawamoto's firm method  
for Kawamoto's firm method



[Click here to access/download](#)

**Animated Figure (video and/or .ai figure files)**

Movie 1\_ 55054\_short.mp4





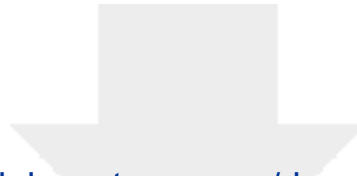


[Click here to access/download](#)

**Animated Figure (video and/or .ai figure files)**

Movie 2a \_ 55054\_ malleus.mp4

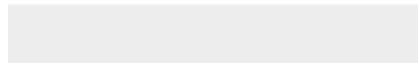




[Click here to access/download](#)

**Animated Figure (video and/or .ai figure files)**

Movie 2b\_ 55054\_Incus.mp4





[Click here to access/download](#)

**Animated Figure (video and/or .ai figure files)**

Movie 3\_ 55054.mp4





1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
[www.jove.com](http://www.jove.com)

# ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s):

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

## ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's


expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name:	Koichi Matsuo		
Department:	Laboratory of Cell and Tissue Biology		
Institution:	Keio University School of Medicine		
Article Title:	Dissection of the Auditory Bulla in Postnatal Mice: Isolation of the Middle Ear Bones and Histological Analysis		
Signature:		Date:	August 15, 2016

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

## Point-by-point response to reviewers' comments

Thank you for your valuable suggestions on our manuscript (JoVE55054). We found your comments extremely helpful in improving and enriching our manuscript. We agree with all the points presented. However, we would like to address one issue differently than you suggested and explain this in a separate sheet at the end.

### *Editorial comments:*

*1. Thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

A professional scientific editor has checked spelling and grammar of the manuscript.

*2. Define all abbreviations before use.*

We defined the following abbreviations:

[3.1.2] paraformaldehyde (PFA)

[3.1.3] ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-2Na).

*3. 3.1.6: What is the series of ethanol dehydrations?*

We now state this series as follows:

[3.1.6] (70%, two changes of 95%, three changes of 100%, each 1 hr).

*4. Formatting:*

*-Define all abbreviations at first occurrence (ie PFA).*

We have defined all abbreviations.

*-3.1.2 – PFA is toxic and requires a caution statement.*

We have added the following:

[3.1.2] Caution: PFA is toxic and should be handled carefully.

*-3.2.4 – Refer to Section 4 rather than “Step 4”. Corrected.*

*-Line 280 – Remove the stray underlined text. Removed.*

*5. Grammar:*

-Copyedit the manuscript for grammatical errors. Such editing is required prior to acceptance, and some errors are noted below:

*-Correct the title and the short abstract so that appropriate articles are used (ie “to isolate the auditory bulla...”).*

We inserted the article “the” in the title and the short abstract:

[Title] Dissection of the Auditory Bulla...

[Short abstract] to isolate the auditory bulla

*-1.5 – “from the mouth” Corrected to “into the mouse”.*

*-3.1.2 – Delete “by sucking it out” Deleted.*

*-3.1.4 – “every other days” Corrected to “every other day”*

*-4.1.1 –Clarify “place the tympanic membrane horizontally to be parallel to”.*

We have changed this wording to:

[4.1.1] 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).

6. Additional detail is required:

*-3.1.6 – Include a citation.*

We cite An et al. (2003) Principles of Embedding and Common Protocols.

*-4.2.1 – Clarify the orientation in text rather than citing only a figure.*

We replaced “according to Figure 4F-G” with:

[4.2.1] so that the neck and transversal lamina of the malleus are perpendicular to the bottom of the embedding dish (Figure 4D-G).

*-4.5 – Include a citation.*

*Include a step at the end of the protocol for downstream analyses and include citations. This step should not be highlighted for filming.*

We have added information relevant to downstream analyses and cited the “Handbook of Histology Methods for Bone and Cartilage” (2003) for histological methods, “Reference 14” for bone labeling, and “Reference 3 for TRAP staining:

[4.5] 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)<sup>3</sup>, or by immunohistochemistry. Undecalcified cryosections are suitable for bone labeling using fluorochromes<sup>14</sup>, alizarin red staining for calcium, and immunofluorescence<sup>42</sup>.



## 7. Results:

*-Describe the data in Figure 5 in more detail. Are there any important features highlighted by the staining?*

We have added the following description:

[Representative results] The malleus attached to the tympanic membrane in the auditory bulla revealed ongoing endochondral ossification at P14 (Figure 5A). For bone labeling, calcein (30 µg/g bodyweight) was peritoneally injected into a P20 mouse, and bulla and capsule were isolated 24 hr 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 <sup>43</sup>. 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).

Horizontal sectioning of the malleal processus brevis (mPB) also shows the cochlea (Figure 5C).

*-Line 238 – A movie (Movie 1) has been cited; however, no movie has been provided for evaluation nor is there a corresponding figure legend for it. Please remove this citation.*  
We now provide Movies 1-3, and inserted citations into protocols.

8. Discussion: Discuss the significance with respect to alternative methods, the limitations, and any troubleshooting/modifications that can be performed.

We now discuss the significance and troubleshooting as follows:

[Discussion] Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14 <sup>50</sup>. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. 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 (styliiform 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.

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.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

*The authors deploy 6 figures to convey a dissection technique of the murine auditory bulla and the middle ear ossicles as well as tissue orientation guidelines for frozen and paraffin sectioning of the middle ear. A comparison of the mouse and human middle ears noting differences and similarities is also presented. The attention to anatomical detail is a particular strength of the manuscript.*

*Major Concerns:*

*Manuscript Title: The auditory bulla and ossicles are dissected but there is no dissection of the otic capsule as such. The title should be refined to state precisely the methods communicated. It would be ideal to include some hint about the histological approach offered as well. Something like: "Dissection of the auditory bulla in postnatal mice: isolation of the middle ear bones and histological analysis in two planes."*

Based on your suggestion, we changed the title to:

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

*1) Abbreviations in the legends: The authors list all of the abbreviations at the end of the legends but use abbreviations before formally introducing the term. The present format makes it difficult for the reader to follow the procedural step without shifting down to the abbreviations and then to the figure; the focus should be on assimilating the information in the figure, not tracking down abbreviations. Define the abbreviations on first use, do so dynamically, and then list only those abbreviations that were not referred to directly at the end of the legend. Done.*

*2) L111: IACUC issue: As written, it appears that the mouse is added to paper towels soaked in Isoflurane, which is not appropriate technique since anesthetic saturated paper towel could come into direct contact with the mouse and irritate mucous membranes causing pain. The mouse should be placed on a platform above the paper*

*towels avoiding direct contact with the isoflurane soaked paper towels. The authors should clarify this critical issue.*

In the revision, we now state the following:

[1.1] Euthanize mice in a jar containing a platform above paper towels soaked in isoflurane or sevoflurane until respiratory ventilation ceases for more than a minute and then perform cervical dislocation. Be careful to avoid direct contact of mice with the soaked paper towels.

*3) L133. This sentence instructs us to dissect out the bulla and capsule together with surrounding tissues. But how? This is effectively the title of the manuscript but the precise dissection technique is not articulated. More descriptive input should be included here. Where do we put our forceps to perform this dissection?*

We described the procedure in the following passage, which is included in the revision:

[1.10] 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, Movie 1). 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.

*4) Section 1: the methodological steps need to be precisely linked to the figures and panels that help the reader understand the step articulated. A major weakness is that the reader needs to figure out for herself what the authors are trying to communicate instead of being led directly to the useful panel. The entire procedural section should carry a figure number and panel at each major instruction. The figures have this detail, but the prose does not lead us there effectively.*

[Protocol] We have inserted figure numbers into each major instructional section.

*Minor Concerns:*

*Abstract: last sentence: what does "various aspects of auditory ossicles" mean here? Be precise in the value of this work.*

[Long abstract] We replaced "various aspects" with "pathological, developmental and evolutionary aspects".

*L64: fix "opto" Corrected.*

*L65-68: This OM discussion is not well linked to the preceding ideas. Work middle ear infection into this paragraph better by perhaps noting how frequently OM brings kids into the ENT office! Make the role of the middle ear vital to the reader; OM is an enormous clinical problem that uses critical clinical resources to address. So your method could be useful for interrogation of animal models of OM.*

Based on the reviewer's comment, we revised the following paragraph of the introduction:

[Introduction] 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 <sup>9</sup>. Mouse models of otitis media could prove useful in understanding the pathogenesis and in developing treatments <sup>10,11</sup>.

*92-96: Do not use "it" serially here. Also, this summary paragraph is precisely the rationale for the new title: isolation of ossicles and histological sections.*

[Introduction] We replaced “Secondly, it demonstrates” with “Secondly, this protocol demonstrates....”

*L115: neck muscle tissue*

[1.2] We inserted the word “neck”, as requested.

*L117: decapitate not "cut off the head". How large are the scissors? Are they sharp? Give us relevant details.*

We now provide relevant details as follows:

[1.3] Decapitate mice at the cervical region using 14-cm sharp surgical scissors.

*L166: how do we remove the stapes? Where do we grab it safely? Which direction do we pull in?*

We now provide the following information:

[2.2.2] Insert a sewing needle (or a marking pin) into the obturator foramen of the stapes and lift up the stapes.

*L181: every other day* Corrected.

*L183: Store in 70% ethanol but did we get from aqueous solution to 70% ethanol by graded ethanols to 70% ethanol? Is the 70% ethanol made in PBS?*

We now provide the following information:

[3.1.5] Optional: Transfer to 70% ethanol through graded alcohol series (30%, 50%, 70% in water).

*L280: identify the posterior cranial fossa in Fig. 1*

[Figure 1] We have now labeled the posterior cranial fossa (pcf).

*L280: there are small black arrows that are unidentified.*

We are sorry but we do not see black arrows in this figure; possibly the reviewer is mistaken about them or could clarify the question.

*L289: Fig. 2 is called "Isolation of the malleus" but it is never truly shown isolated from the middle ear until Fig. 6. Make the figure title accurate. Dissection of the malleus?*

We have now changed the figure title to:

[Figure 2 title] Dissection of the malleus.

*L304: Dissection of the incus and stapes?*

We have now changed the figure title to:

[Figure 3 title] Dissection of the incus and stapes.

*L307: the scissor icon is hard to see; outline in black.*

[Figure 3B] We have replaced the icon with a “needle tip” icon.

*L313: the dashed lines referred to are not present. The arrowhead is not defined? Also, why are the bullae in A,D,F green? The green middle ear bones in B,C,E,G need to be labeled in some way so we can use this anatomical information to understand the required orientation. Also, the microCT image should be labeled so we have some common anatomical landmarks for appreciating the orientation required. We need to be able to repeat this method and get the results shown in the representative data section and this figure is not properly rendered to achieve this goal.*

We have extensively revised the figure legend and improved Figure 4. We now also state that the bullae are green because the photo was taken with a color filter:

[Figure 4 legend] **Orienting the auditory bulla and capsule during embedding for longitudinal (parasagittal, A-C) and horizontal sectioning (D-E) 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 (styliiform process) was removed. i, incus. (C) Top view: micrograph (taken with a color filter). AC, anterior (superior) semicircular canal; Sf, subarcuate fossa; Sp, styliiform 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  $\mu$ m, as previously described <sup>7</sup>

*L326: Add to each panel the animal age and method of preparation. P6 Longitudinal Paraffin added to panel A for example.*

[Figure 6] Information relevant to age, method, orientation, and staining (labeling) has been added to each panel.

*L333: Remarkably, the mouse ossicles in panel B are not mentioned and should be. Presumably they were imaged at the same magnification as the human ossicles.*

We have now added the following information:

[Figure 6B legend] Ossicles of P31 mouse are imaged at the same magnification as that used for human ossicles.

*L276: Panels E and F should be oriented so that their angles match precisely. Cochlea might be abbreviated CO and CC could be used for common crus.*

[Figure 1] We have added an orientation symbol to Panel F (rather than rotating the image). We now abbreviate cochlea as Co.

*L289: Adjust the brightness, contrast, and midtones of Panel H so there is better delineation of the 3 ossicles.*

[Figure 2] We have replaced Panel H with a new photo to better delineate the ossicles.

**Reviewer #2:**

*Manuscript Summary:*

*This is a well-written description of how to isolate the bulla and ossicles. The information about embedding the bulla for sectioning could be very useful but needs some clarification.*

*Major Concerns:*

*-The cryostat images (Fig 5B,C) are not very nice. Figure 5B was stained with alizarin complexone but this looks like H & E. A better example should be shown or Alizarin red should be used. The difference in angle between 5A and B is also quite minor (we can see the orbicular apophysis and part of the manubrium of the malleus in both indicating a similar angle). Are the authors sure they have orientated the samples correctly?*

[Figure 5B] Based on your comments, we replaced Alizarin red staining with calcein bone labeling. We also now provide information relevant to orientation in each panel of Figure 5.

*-What are the planes of section referring to? It would be helpful if they were the positions of the bulla in the head, which they don't appear to be.*

In the revision we have improved explanation of the planes in both Figures 4 and 5.

*What is the benefit of dissecting the bulla from the head before sectioning? This should be mentioned.*

We now discuss the benefit of dissecting the bulla before sectioning in the following passage:

[Discussion] Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14<sup>50</sup>. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. 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 (styliiform 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.

*-The orbicular apophysis is not the processus brevis, this is a different structure (see papers by Mason).*

Please see separate response sheet at the end.

*Minor Concerns:*

*-Method wise, I would suggest they don't jump from PBS to 70% Ethanol but use a graded series instead.*

We now provide the following information:

[3.1.5] Optional: Transfer to 70% ethanol through graded alcohol series (30%, 50%, 70% in water).

*-Histoclear should be given as an alternative to xylene, as xylene (due to its carcinogenic nature) is not allowed in many labs in Europe.*

We now provide the following information:

[3.1.6] Optional: Substitute xylene with Histo-Clear, which is a non-toxic and non-flammable histological clearing agent.

*-The dotted lines outlining the different components of the EAM are based on what? Are the authors sure they have the delineations correct?*

We replaced the photo in question and have improved the Figure 2A legend to read:

[Figure 2A legend] 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.

*-The authors mention that it is difficult to isolate the bulla before P12. This should be correlated with known descriptions of bulla development (Richter et al etc). The authors should also refer to the timing of cavitation.*

We now discuss this point as follows:

[Discussion] First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14<sup>50</sup>. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. 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.

*-There are a few typos in the Table of materials*

*For example Eosin, containers, stainless, Haematoxylin.*

[Table of materials] These have now been carefully checked.

### **Reviewer #3:**

#### *Manuscript Summary:*

This was a well-written and generally clear paper which explains how to access and view a small but important part of the anatomy of mice. I can see it being useful to auditory researchers who are starting in this field. The good-quality photographs were useful aids which illustrate the techniques.

#### *Major Concerns:*

My three main comments for improvement of this paper are all relatively minor and easy to implement:

*1) In the methodology section, the step-by-step instructions should be explicitly linked with the figure panels showing those stages, where applicable. This would make it easier for the reader to see where he/she is in the process.*

Done.

*2) Although the developmental biology literature has used the term "processus brevis" to mean "orbicular apophysis", this is a mistake which has simply been repeated in several papers. As explained in more detail below, the term "processus brevis" should be removed from this paper (as the true processus brevis is not referred to or illustrated) and the text and figures should refer consistently to the orbicular apophysis. Please see separate response sheet below.*

*3) Micro-CT imaging is mentioned in this paper and CT-derived images are presented in Figure 4. However, the paper does not describe how these scans were made and processed.*

We now provide micro-CT method:

[Figure 4 legend] Micro-CT images were obtained at a voxel resolution of 5  $\mu\text{m}$  as previously described (Kanzaki et al, 2011).

*Minor Concerns:*

*Line 42: The auditory ossicles are usually the smallest bones in the body, but this is not true in all mammals. See, for example, the relatively enormous ossicles of golden moles, as discussed in Mason, 2013 (already on the reference list).*

We have now changed the Abstract and the Discussion to state:

[Abstract] In most mammals, ... the smallest bones

[Representative Results] 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 (Mason, 2013).

*Line 64: "optoacoustic" should read "otoacoustic". Corrected.*

*Line 70: Although it is true to say that most of the auditory ossicles are formed by endochondral ossification, this is not true of the goniale, as the authors acknowledge on line 271. The goniale is considered a part of the malleus. Here's a reference:*

*Rodríguez Vázquez, J.F., Mérida Velasco, J.R. & Jiménez Collado, J. (1991) A study of the os goniale in man. Acta Anatomica 142: 188-192.*

We have now inserted the following information:

[Introduction] except for the goniale part of the malleus (Rodríguez Vazquez, 1991; Tucker, 2004)”.

*Line 109: As well as putting a whole figure reference here, particular numbered steps should be explicitly linked to the appropriate parts of Figure 1. For example, step 1.7 seems to equate to panel 1A, and it should say so. This applies also to Figures 2 to 5. See major comments. Corrected.*

*Line 112: "respiration" should be replaced with e.g. "respiratory ventilation". Corrected.*

*Line 131: "further underneath (lateral to)". "Underneath" would normally be taken to mean "ventral to", so how does this equate to "lateral to"? This needs rephrasing.*

[1.8] We have now replaced “underneath” with “lateral to”.

*Lines 146, and 254-5. The term "processus brevis" is NOT synonymous with "orbicular apophysis": the two are different processes (see major comments above).*

*To expand on this, the term "processus brevis" has been widely used - wrongly - in the developmental biology literature to refer to a prominent process of the mouse malleus, the one labelled in the current paper (e.g. in Fig. 6A). However, this term seems to have been borrowed, incorrectly, from the human anatomical literature. The "processus brevis" in humans is also known as the "lateral process", a projection at the base of the manubrium. Mice have this too. However, the authors are describing and labelling in their figures the "orbicular apophysis", a different process which has no equivalent in humans. See Mason (2013) for a discussion and diagrams. All uses of "processus brevis" in this paper should be changed to "orbicular apophysis", in text and figures. Mason (2013) can be cited as a reference, to avoid confusion.*

We understand this point. However, we would like to use both “processus brevis” and “orbicular apophysis”. Please see the separate sheet relevant to this issue below.

*Line 173, 196, 235: "Styliform process" could perhaps be mistaken by those familiar with the human ear as the "styloid process", but the authors are actually referring to the processus styloformis, a little process of bone contributing to the Eustachian tube. This should be made clearer. N.B. "styliform" is an adjective so always needs to be paired with a noun (i.e. always write "the styliform process", not just "the styliform").*

[1.10] We have added, “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.”

[3.1.2],[ 3.2.2] “the styliform” was replaced by “the styliform process”.

*Line 181: change "every other days" to "every other day".* Corrected.

*Lines 215-6: The method discussed here suggests that the tympanic membrane should be parallel to the bottom of the embedding dish, in order to get sagittal sections (N.B. this should be "parasagittal", i.e. parallel to the sagittal plane down the midline). This seems to imply that the tympanic membrane in mice is oriented in the parasagittal plane. However, it is in fact inclined at an angle of maybe 20-30 degrees to the vertical, according to the diagram in van Kampen (1905: p.553). Has this orientation been taken into consideration?*

*van Kampen, P.N. (1905) Die Tympanalgegend des Säugetierschädels. Gegenbaurs Morphologisches Jahrbuch 34: 321-722.*

[4.1] Corrected to “Longitudinal (parasagittal) sectioning of the malleus”

We also added, “Note that the tympanic membrane and malleus are inclined at an angle at approximately 30 degrees to the vertical in the head (Figure 4A, Fig. 59 in Kampen, 1905).”

*Lines 222-4: Along similar lines to the above, is the manubrium really exactly horizontal? I suggest that it should be made clear that the planes referred to (parasagittal and horizontal) are only approximate. "Vertical to the bottom" should presumably read "perpendicular to the bottom".*

We explained that the planes are relative to the malleus in Figure 4.

[4.1.1] Note that the tympanic membrane is inclined at an angle at 20-30 degrees to the vertical in the mouse head (Figure 4A, Fig. 59 in Kampen, 1905).

[4.2.1] [4.3.1] Corrected to "perpendicular to the bottom".

Line 238: Movie 1 is not included within the material provided to me as a Reviewer.

We now provide Movies 1-3, and provide references to them.

*Lines 253, 262: Birds have wings of many different shapes and aspect ratios, so this is not a very useful description! The sword analogy is better.*

[Representative results] The “bird wing-like” was replaced by “gliding-seagull-wing-like”.

*Lines 257, 271: "...where the gonium is located". The goniale (which is the more usual term for this ossification) forms part of the anterior process, but in my experience it is impossible to determine where the goniale fuses with the endochondral parts of the malleus. Perhaps these two passages should be rephrased, or alternatively the authors could describe and show more clearly in the diagrams where they believe the union to be.*

We clarified this point in the Figure 2H legend:

[Figure 2H legend] “Go, goniale (fused to the malleus and the tympanic ring)”

*Line 263: The anatomical axis is the line through the anterior process of the malleus and the short process of the incus, as described here. However, many vibrometric studies have shown that this rarely coincides with the true axis of rotation, especially in human ossicles. Some of the references referred to in the text discuss this; see below for another.*

Willi, U.B., Ferrazzini, M.A. & Huber, A.M. (2002) *The incudo-malleolar joint and sound transmission losses. Hearing Research* 174: 32-44.

We now provide the following information:

[Representative results] In human ossicles, vibrometric studies reveal that the incudo-malleolar joint is mobile rather than functionally fixed (Willi, 2002).

Line 265: should read "and the two are almost parallel". Corrected.

Line 265: Maier & Ruf (2016) did not examine mouse and human ossicles, so it is not clear how this paper is relevant here. The authors should go to the original sources for their references.

We have now replaced Maier & Ruf (2016) with Fleischer (1978).

Lines 265-6: Noting my earlier comments about this, this statement is incorrect - it is the orbicular apophysis (not the processus brevis) which "is a prominent semi-spherical protrusion in mice, while in humans it is not apparent". The true processus brevis is present in both, representing the projecting root of the manubrium at the point where it inserts into the tympanic membrane at the opposite end to the umbo.

We agree with this statement. Please see the separate sheet relevant to this issue.

Line 270: There is no need to put quotation marks around "lamina" here: this is a well-established descriptive term for mouse malleus structure. It is usually called the transversal lamina.

We now removed quotation marks around "transversal lamina"

Lines 271-2: It seems odd to say that the tympanic bone anchors the malleus to the skull, when the tympanic bone is normally taken to be part of the skull!

We have now deleted "both of which anchor the malleus to the skull".

Line 277: "Medial surface of the right skull" should read e.g. "medial surface of the right half of the bisected, skinned head".

Corrected.

Line 279: There is only a single arrowhead pointing to the "dorsal crest" in panels 1D and 1E. Please include second arrowheads at the other end of the crest, because at

*present there is more than one crest-like structure visible in panels 1D and 1E which the arrow might be indicating.*

[Figure 1DEF and Figure 4A-F] We have now added the requested second arrowhead.

*Line 285: a period is erroneously underlined here. Corrected.*

Line 290: Specify whether this is a left or right bulla.

The word “right” has been inserted into the legend:

[Figure 2A legend] Ventrolateral view of a right auditory bulla and capsule.

*Line 300: Be consistent in the use of "gonium" or "gonial" throughout the paper ("gonial" or "goniale" is the commoner term which I personally would prefer). Also - can this actually be distinguished in panel 2H? I don't think it can.*

We use the term “goniale” throughout the revised manuscript. The Figure 2H legend was changed to, “Go, goniale (fused to the malleus and the tympanic bone)”.

*Line 301: Make it clear that the air-bubble is within the middle ear cavity, seen through the tympanic membrane.*

We revised the legend to read:

[Figure 2C legend] Arrow, air bubble in the middle ear cavity seen through the tympanic membrane.

*Line 306: We are asked to note that "the short crus of the incus is fixed by the posterior ligament", but this ligament is not visible in any of the photomicrographs here.*

We have now clarified this point in the Figure 3A legend:

[Figure 3A legend] Note that the short crus (iCB, Crus breve) of the incus (i) is fixed by the posterior ligament (not shown).

Line 313: Make it clear whether we are looking at a left or a right bulla here.

[Figure 4AD legend] The word “right” is now inserted.

*Line 314: There is no explanation anywhere in this article about how CT images were obtained, reconstructed or coloured (see major comments). A short paragraph at least is required!*

We have added the following sentence to the Figure 4 legend and cited a reference:.

[Figure 4 legend] Micro-CT images were obtained at a voxel resolution of 5  $\mu$ m as previously described (Kanzaki, 2011).

*Line 335: Make it clear that Fig. 6B also shows the mouse ossicles, to scale. It should also explain in the caption what the dotted lines and the curved arrow represent.*

[Representative results] We have now added “at the same magnification.”

[Figure 6B legend] Ossicles of P31 mouse 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).

*Lines 338, 342: I don't see the muscular process (mp) labelled in the figure.*

[Figure 6 legend] We now indicate “black asterisk (muscular process of the malleus)” and later “white asterisk (muscular process of the stapes)”.

*Line 339: As referred to previously, the label "mPB" actually points to the orbicular apophysis, which is NOT synonymous with the processus brevis. I have never heard the term "tuber mallei", which I suggest is deleted.*

We have now deleted “Tuber mallei”. Please see the separate sheet relevant to mPB versus orbicular apophysis issue.

Lines 351-355: It is not clear enough here whether the middle ear cavity of mice of this age is filled with fluid normally, and "cavitation" refers to the natural event whereby the fluid is replaced with air, or whether the fluid is appearing as a post-mortem artefact and "cavitation" is simply the process of bubbles forming therein. Please be clearer about when the fluid in the middle ear is removed, in ontogeny.

We now clarify the point in the following:

[Discussion] Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14<sup>50</sup>. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. 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 (styliiform process) rather than trying to do so in the unisolated bulla.

*Fig. 1F: It is not clear what the styliiform process label is actually indicating here, since no process is visible.*

We have replaced the Figure 1F photo and clearly labeled the styliiform process (Sp).

*Figures 2, 5, 6: All references to the "processus brevis" in captions and figures should be changed to "orbicular apophysis" (see above).*

[Please see the separate sheet about this issue.](#)

*Fig. 2D: The arrow labelling the malleus head appears to be pointing to the base of the anterior process. The head is nearer to the articulation with the incus.*

[Corrected.](#)

*Fig. 3: The arrow in panel B and the cross in panel C are not explained in the caption.*

[Both the arrow and the cross are now explained:](#)

[Asterisk, muscular process of the stapes.](#)

[X indicates the cut end of the stapedial artery \(SA\).](#)

*Fig. 6A: As per my previous comment, the label attached to the head of the mouse malleus seems to be too far towards the anterior process. I would regard the swollen region next to the articulation with the incus as the true head, i.e. somewhere to the right of the current label.*

*Materials spreadsheet: "tangsten" should presumably read "tungsten", "steinless containers" should read "stainless containers", "rotater" should be "rotator", "haemotoxyrin" should be "haemotoxylin", "eodin" should be "eosin", "vender" should be "vendor", "silan" should be "silane".*

[All of these errors have been corrected in the revision.](#)



## **“Processus brevis” versus “orbicular apophysis”**

Both Reviewers #2 and #3 asked that the term “processus brevis” be replaced by “orbicular apophysis”. We agree that the orbicular apophysis is a correct term. However, we would like to 1) use the term “processus brevis” in the paper for the following reason, and 2) explain to the readers in the Discussion why we prefer the term.

### **The term is established:**

“Processus brevis” has been used to indicate the orbicular apophysis for more than two decades particularly in the field of mouse developmental biology. For example, there were 2,640 citations of papers published during the 90's that used this terminology.

Rijli et al (1993) Cell [439 times cited]

Satokata et al (1994) Nature Genetics [840 times cited]

Martin et al (1995) Genes & Development [212 times cited]

Rivera-Pérez et al (1995) Development [210 times cited]

Yamada et al (1995) Development [206 times cited]

Houzelstein (1997) Mechanisms of Development [113 times cited]

Depew et al (1999) Development [230 times cited]

Xu et al (1999) Nature Genetics [394 times cited]

We believe that the current manuscript we submit to JoVE should not use terminology different from these publications.

"Therefore, we would like to use the term *processus brevis* in the paper and then explain why we prefer that term at the end of the “Representative results” section as follows:

[Discussion] 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<sup>16,20,22-25</sup>. “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 Fig. 6B, medial view). In mice, the lateral process is also a projection of the manubrium at

the opposite end to the umbo <sup>48</sup>. The pars flaccida of the tympanic membrane is above the lateral process of the malleus. Orbicular apophysis is not apparent in the human malleus.