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TITLE:

Dissection of the Endolymphatic Sac from Mice

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SUMMARY:

This study describes how to dissect the endolymphatic sac from the inner ear of the mouse at different ages. The result of a similar dissection is shown in an *Slc26a4*-null mouse model of enlargement of the endolymphatic sac. A transgenic mouse with a fluorescent reporter expressed in the endolymphatic sac is presented as a model to readily visualize the endolymphatic sac, and educational tool.

ABSTRACT:

The study of mutant mouse models of human hearing and balance disorders has unraveled many structural and functional changes which may contribute to the human phenotypes. Although important progress has been done in the understanding of the development and function of the neurosensory epithelia of the cochlea and vestibula, limited knowledge is available regarding the development, cellular composition, molecular pathways and functional characteristics of the endolymphatic sac. This is, in large part, due to the difficulty of visualizing and microdissecting this tissue, which is an epithelium comprised of only one cell layer. The study presented here

describes an approach to access and microdissect the endolymphatic sac from the wild-type mouse inner ear at different ages. The result of a similar dissection is shown in a pendrin-deficient mouse model of enlargement of the vestibular aqueduct. A transgenic mouse with a fluorescent endolymphatic sac is presented. This reporter mouse can be used to readily visualize the endolymphatic sac with limited dissection and determine its size. It can also be used as an educational tool to teach how to dissect the endolymphatic sac. These dissection procedures should facilitate further characterization of this understudied part of the inner ear.

INTRODUCTION:

The mammalian inner ear consists of the cochlea, the saccule, the utricle, three semi-circular canals, and the endolymphatic sac (**Figure 1A**). These organs comprise a continuous, fluid-filled epithelium called the membranous labyrinth, with adjacent organs connecting directly or through small canal structures such as the ductus reuniens, saccular duct, utricular duct, or endolymphatic duct. Compared to other organs of the inner ear, the endolymphatic sac has unique characteristics. First, it lacks sensory epithelial cells. Instead, the endolymphatic sac has cells specialized for ion transport. Second, although the membranous labyrinth is enclosed in the bony labyrinth, the endolymphatic sac is an exception, protruding partially out of the petrous bone and into the intracranial cavity. This morphology appears to be highly conserved during evolution of the inner ear. Third, the endolymphatic sac is the first structure to develop from the otocyst at an early embryonic stage prior to formation of other organs. In addition several pathologies have been associated with an enlarged endolymphatic sac or an abnormal endolymphatic compartment. The presence of pathogenic variants in *SLC26A4* (also known as the pendrin gene) leads to a comparatively common form of sensorineural hearing loss, caused by the enlargement of the endolymphatic sac associated with the presence of an enlarged vestibular aqueduct (EVA)[1]. When associated with goiter, it is refered to as Pendred syndrome[2,3]. Meniere's disease is also thought by some to be associated with abnormality of the endolymphatic compartment (hydrops)[4]. The unique features of the endolymphatic sac and pathologies associated with a change of its morphology are consistent with a critical role for development and maintenance of the inner ear.

Despite its importance, knowledge is still limited regarding the development, cellular composition, molecular pathways and functional characteristics of the endolymphatic sac. This is at least partly due to the difficulty of visualizing and microdissecting this tissue. The endolymphatic sac is a pouch-shaped structure, composed of a single layer of epithelial cells, which is often translucent and very difficult to identify out of the conjunctive tissue which surrounds it by light stereomicroscopy. Although a small number of researchers have developed whole-mount dissection techniques and published their experimental findings[5-10], there is no publication focusing on the technical details of this procedure.

In this study, the microdissection approaches developed to access and isolate the endolymphatic sac from the wild-type mouse inner ear at different ages, in whole-mount, is described. The results of a similar dissection is shown in a mouse model, lacking expression of SLC26A4, which has enlargement of the endolymphatic duct and sac. A mouse line with a transgene encoding a Cre recombinase expressed in the endolymphatic sac is reported. In presence of a fluorescent

reporter of Cre expression, here tdTomato, the fluorescence can be used to readily visualize the endolymphatic sac with limited dissection and to obtain information about its size. This reporter mouse line can also be used as an educational tool to practice and perfect endolymphatic sac dissections. The ability to dissect endolymphatic sac tissue should facilitate further characterization of this understudied but essential component of the inner ear.

PROTOCOL:

All animal experiments and procedures were performed according to protocols approved by the Animal Care and Use Committees of the National Institute of Neurological Diseases and Stroke/National Institute on Deafness and Other Communication Disorders (#1264) and the Institutional Animal Care and Use Committee at Tokyo Medical and Dental University (A2020-058A).

NOTE: C57BL/6J mice (stock number no. 000664), *Pds*$^{-/-}$ also known as *Slc26a4*$^{-/-}$ mice (129S-*Slc26a4*$^{tm1Egr/AjgJ}$, no. 018424)[11], B6;CBA-Tg(ATP6V1B1-Cre)$^{1Rnel/Mm}$ mice[12] and Ai9(LSL-RCL-tdT) (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$, no. 007909) mice[13] are available from the Jackson Laboratory and upon request. All genotyping procedures have previously been described. B6;CBA-Tg(ATP6V1B1-Cre)$^{1Rnel/Mm}$ mice previously backcrossed to strain C57BL/6J for at least five generations, were bred for experiments. Animals of both sexes can be studied with this approach without an obvious difference. The first day after overnight mating is counted as embryonic day 0.5 (E0.5).

1. Harvest and fixation of the intact inner ear in the skull

1.1. Euthanize experimental animal or time-pregnant dam using an institutionally approved procedure, for example, using CO_2 inhalation. Work under a fume hood if possible, to avoid or minimize the spread of mouse allergens. After verifying the lack of response to painful stimuli such as paw or tail pinches, decapitate the animal. For a time-pregnant dam, cervical dislocation can be used as an alternative to decapitation if its inner ears are not used for further experiments.

1.2. Harvest the embryos by laparotomy[14] using artery scissors with a ball tip to protect the embryos during the process. Manipulate the embryos using ring forceps. Place the uterine horns in 4 °C PBS on ice and isolate each embryo as previously described[15] before decapitating them.

1.3. Bisect the head from back to front: first incise the top of the head by first incising the skin, then the top of the skull, incising about one third of it with each cut so there is no bending of the skull. Then cut bottom of the skull at once.

NOTE: For embryos, the use of a sharp blade, rather than scissors, is advised. It is critical that, at all ages, the skull is not deformed while bisecting it.

1.4. Without touching or bending the skull, carefully remove the hemi-brain to reveal the bony labyrinth (**Figure 1B, Supplementary Figure 1A-B**).

1.5. For mice postnatal day 5 or older, transect the external auditory canal and pull the skin forward towards the nose. Trim the skull around the inner ear (**Supplementary Figure 1C**).

1.6. Transfer the two hemi-skulls of each animal with the inner ear, as intact as possible, to a glass vial or tube containing 10 mL of 4 °C 4% paraformaldehyde (PFA) diluted in 1x PBS and keep on ice under a fume hood.

NOTE: Do not try to remove the inner ear from the skull at this step as this may damage the endolymphatic duct and sac while doing so.

1.7. Incubate for 1 h at 4 °C on a rocking shaker. If working with tissue from an animal with a fluorescent reporter, maintain the tube containing the specimen in aluminum foil or in a box to minimize exposure to light.

NOTE: If interested in studying microtubules and associated molecules, tissue fixation should be performed at room temperature (RT) with RT fixative.

1.8. Discard the PFA in an appropriate container in a fume hood. Wash three times with 10 mL of 1x PBS for 15 min each time on a rocking shaker at RT to eliminate the fixative before dissection.

2. Endolymphatic sac microdissection

2.1. Transfer each inner ear preparation to a 35 mm tissue culture dish containing 1x PBS.

2.2. Place the preparation in the dish so that the root of the VIIIth cranial nerve is oriented up. Hold the tissue at the position of cochlea using a #4 forceps (**Figure 1C**).

2.3. Identify important landmarks: the vestibular aqueduct, the anterior and posterior semicircular canals, common crus, and sigmoid sinus (vein) (**Figure 1D**).

NOTE: The endolymphatic sac in the wild type mouse is translucent and hard to visualize. For successful microdissection of the endolymphatic sac, it is important to understand its anatomy and localization (**Figure 1E**).

2.4. Incise the dura mater and vestibular aqueduct, as well as the underlying connective tissues surrounding the endolymphatic sac, using a 27 G needle on a 1 mL syringe (**Figure 1D,F**).

NOTE: For an adult mouse, decalcification of the preparation using 10% EDTA (pH 7.4) before microdissection is useful, but not essential, to facilitate incision of the vestibular aqueduct. For dissection of opened endolymphatic sac older than postnatal day 5, it is recommended to position the incision line on the vestibular aqueduct slightly anterior to make an incision into the endolymphatic sac lumen (**Figure 2A**).

2.5. Hold the connective tissue located lateral to the endolymphatic sac with forceps and pull up the tissue to peel it from the temporal bone (**Figure 1G**).

2.6. Carefully remove any remaining debris. The preparation usually includes the endolymphatic sac epithelium, surrounding connective tissues, a portion of the vestibular aqueduct, and the sigmoid sinus (**Figure 1H,I**). If necessary, separate the endolymphatic sac epithelium from surrounding tissues (**Figure 1J**).

2.7. (Optional) For dissection of the opened endolymphatic sac, hold the stem part of the preparation so that the cross-section of the lumen can be observed. Insert a 27 G needle into the lumen and move it to cut the endolymphatic sac into two sheets (**Figure 2B,C**). Hold the edge of each sheet-like tissue with forceps and separate them from each other (**Figure 2D-F**).

3. Immunohistochemistry

3.1. After microdissection, use #5 forceps to transfer endolymphatic sacs into a 9-well spot glass plate.

3.2. Using the microscope to make sure the endolymphatic sacs remain untouched, use a 200 µL pipette to remove all 1x PBS except one drop containing the tissue. Add 200 µL of permeabilizing and blocking solution (1x PBS with 0.15% Triton X-100 (PBS-TX) and 5% bovine serum albumin (BSA)) and incubate for 1 h at RT on an orbital shaker.

3.3. After removing all blocking solution except one drop containing the tissue, add 200 µL of primary antibodies and incubate overnight at 4 °C. Anti-pendrin antibodies[6] diluted 1:1000 in blocking solution can be used to identify the mitochondria-rich cells of the endolymphatic sac, although a faint expression is also seen in a subset of ribosome-rich cells[16] (**Figure 5**).

3.4. After three washes in 1x PBS for 15 min at RT, incubate with secondary antibodies. For the labeling presented here, incubate 200 µL of fluorescent dye-conjugated secondary antibodies, diluted from the commercial stock at 1:500 in blocking solution, with the tissue on a rocking platform for 1 h at RT in the dark. Fluorescent dye-conjugated phalloidin, which recognizes β-actin (ACTB), can be used to highlight the presence of the endolymphatic sac and duct as well as the conjunctive tissue around them (**Figure 5A, C, F-G**).

3.5. Mount endolymphatic sacs between a glass slide and coverslip using an antifade mounting medium with DAPI. After letting the slides dry protected from light for 1 h at RT, apply transparent nail polish at the junction between coverslip and glass slide to seal them and limit air bubble formation. If trying to visualize the differential distribution of proteins at the apical *versus* the basolateral surface of the epithelial cells, it is recommended to open the sac during the mounting step by incising an edge and opening it.

3.6. Image using a confocal microscope. First visualize endolymphatic sac preparations are with a 10x objective to provide a global view of the sac labeling (**Figure 5A-D**). Then use a 63x objective to gain a detailed view of the distribution of the protein of interest in mitochondria- and ribosome-rich cells of the endolymphatic sac (**Figure 5F-G**).

REPRESENTATIVE RESULTS:

Each step of this microdissection of a wild type endolymphatic sac from a postnatal day 5 (P5) mouse is detailed in the associated video and snapshots of the key steps of this dissection and opening of the endolymphatic sac are presented in **Figure 1** and **Figure 2**.

Representative results of the dissections of the endolymphatic sac with tdTomato fluorescence at embryonic day 16.5 (E16.5), P5 and P30, following this protocol are presented in **Figure 3** and in **Supplementary Figure 1** and **Supplementary Figure 2**. Using a dissection microscope with fluorescence, the endolymphatic sac can be readily visualized in the skull after removal of the brain in mice carrying the transgene Tg(ATP6V1B1-Cre) in the presence of the reporter of Cre expression: Ai9 (LSL-RCL-tdT) (see **Figure 3A-B**, and **Supplementary Figure 1** for images captured in incident light and tdTomato fluorescence of the endolymphatic sac at different stages of the dissection). In these mice, Cre is expressed as a mosaic in cells all along the endolymphatic sac and duct (**Figure 3**, see also **Supplementary Figure 2** for visualization of the endolymphatic sac with *versus* without fluorescent reporter). Although the expression of this reporter is not specific for the endogenous expression of *Atp6v1b1*, which is restricted to the mitochondria rich cells of the endolymphatic sac[16], one of the two cell types of this epithelium (**Figure 5E**), it is a helpful aid to identify and dissect the endolymphatic sac.

Inner ears dissected from a mouse model for enlargement of the vestibular aqueduct are presented in **Figure 4**. As compared to control littermates, the endolymphatic sacs and ducts of mice deficient for SLC26A4 (pendrin) are enlarged.

Representative images of immunohistochemistry of intact endolymphatic sac with and without conjunctive tissue are shown in **Figure 5**. Results of immunohistochemistry of opened endolymphatic sac of a P5 wild type mice is also presented (**Figure 5F-G**). The endolymphatic sac is composed of a single layer of epithelium folded in a pouch-shaped structure, containing in its lumen endolymph. This epithelium consists of two cell types, mitochondria-rich cells and ribosome-rich cells (**Figure 5E**). A whole-mounted endolymphatic sac will be flattened because endolymph is no longer present. The sac will appear as two intertwined layers of epithelium, making it difficult to determine subcellular localization of proteins expressed in this epithelium. By opening the endolymphatic sac, the single layer epithelium can be readily visualized and the relative distribution of the proteins of interest, with respect to the endolymphatic sac lumen, can be determined more definitively. A potential apical (luminal, where endolymph would be) *versus* basal enrichment of these proteins can be more accurately determined. As an example, SLC26A4 is enriched on the apical side of mitochondria-rich cells (**Figure 5F**).

Figure 1. Whole-mount dissection of the endolymphatic sac of a wild type mouse at post natal day 5 (P5).

(A) Schematic of the membranous labyrinth of a developed mouse inner ear (right). The location of the endolymphatic sac, and the cochlea and vestibular structures are indicated. This figure has been modified from Honda et al.[16].
(B) Mid-sagittal section showing the left hemi-skull. The location of the endolymphatic sac is indicated (arrow). (O, occipital; F, frontal)
(C) Otic capsule along with the squamous part of the temporal bone.
(D) Important landmarks overlaid onto the dotted box in panel C. (ES, endolymphatic sac; ED, endolymphatic duct; VA, vestibular aqueduct; ASC, anterior semicircular canal; PSC, posterior semicircular canal; CC, common crus; SS, sigmoid sinus)
(E) Schematic illustration of the cross-section along with the continuous white line on panel D through the endolymphatic sac. The proximal side of the endolymphatic sac is covered by a bony canal called the vestibular aqueduct, along with the endolymphatic duct. In the distal side, the endolymphatic sac, surrounded by conjonctive tissue (CT), extends and protrudes to the outside of the bony labyrinth and is sandwiched between the dura mater (DM) and sigmoid sinus with the squamous part of the temporal bone (TB).
(F) A fine needle is used to cut around the endolymphatic sac (following the dotted yellow lines on panel D).
(G) The preparation is carefully removed from the temporal bone by holding the tissue at the position shown by the asterisk (*, also shown in panel E) and peeling it up.
(H) An isolated whole endolymphatic sac with surrounding tissues.
(I) Schematic version of this isolated endolymphatic sac with surrounding tissues.
(J) Isolated endolymphatic sac without surrounding tissues.
Scale bars: 2 mm (B), 1 mm (J).

Figure 2. Dissection of the opened endolymphatic sac of a P5 wild type mouse.
(A) On step 2.4, the incision line on the vestibular aqueduct is positioned slightly anterior (red dotted line) to make an incision into the endolymphatic sac lumen.
(B) Hold the stem part of the preparation, insert a 27-gauge needle into the lumen, and move it to cut the endolymphatic sac into two sheets.
(C) Schematic illustration of panel B.
(D) Hold the edge of each sheet with forceps and separate them from each other.
(E) Schematic illustration of panel D.
(F) The endolymphatic sac is separated into two sheets, including the epithelium and the surrounding tissues.

Figure 3. Dissection of *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ mice endolymphatic sac at E16.5, P5, and P30.
All images presented show tdTomato fluorescence obtained using a stereomicroscope with a 1 x objective equipped for detection of tdTomato fluorescence.
(A, B) Mid-sagittal section of the skull of a E16.5 *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ mouse before (A) and after (B) half-brain removal. tdTomato fluorescence outlines the position of the inner ear. The endolymphatic sac is readily visible even without dissection (arrowhead).
(C, E, G) Isolated inner ears from E16.5, P5 and P30 mice, respectively.
(D, F and H) Higher magnification images of the corresponding microdissected endolymphatic

sacs and ducts. At P30, the endolymphatic duct is encapsulated in bone making it particularly difficult to isolate. Scale bars: 2 mm (A, B, C, E, G), 500 μm (D, F, H)

Figure 4. Gross anatomy of the otic capsule with an enlarged endolymphatic sac.
Inner ears from *Slc26a4*$^{+/-}$ (left) and *Slc26a4*$^{-/-}$ (right) mouse littermates at P105. The vestibular aqueduct and endolymphatic sac and duct (black dotted lines) are enlarged in the *Slc26a4*$^{-/-}$ mouse as compared to those in a *Slc26a4*$^{+/-}$ mouse. Scale bar: 2 mm.

Figure 5. SLC26A4 expression of mitochondria-rich cells in the endolymphatic sac epithelium at E16.5 and P5.
(A, B) Isolated endolymphatic sac from E16.5 mouse labeled with anti-SLC26A4 antibody (green) and phalloidin which labels β-actin (ACTB, red).
(C) A low-magnification image of opened endolymphatic sac from P5 mouse labeled with an anti-SLC26A4 antibody (green). Phalloidin (ACTB, red) can be used to highlight the presence of the endolymphatic sac as well as the conjunctive tissue around it.
(D) Isolated endolymphatic sac labeled with an anti-SLC26A4 antibody (green).
(E) Schematic illustration of endolymphatic sac epithelium highlighting the presence of two cell types: the mitochondria-rich cells, which apical surface is covered with microvilli, and the ribosome-rich cells.
(F-G) High-magnification maximum intensity projections images of endolymphatic sac epithelium at P5, after the endolymphatic sac was opened and labeled with an anti-SLC26A4 antibody (green) and phalloidin (ACTB, red). A representative image at the apical membrane level is shown in G, and a reconstructed cross-section from z-stack at the level of the punctate white line is shown in F. The nucleus of the cells is labeled with DAPI (blue).
Scale bars: 100 μm (A, B); 200 μm (C, D) ; 20 μm (F, G).

Supplementary Figure 1. Incident light and corresponding tdTomato fluorescence images of a P5 *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ mouse inner ear at three steps of the dissection.
The endolymphatic sac and duct can be readily visualized using tdTomato fluorescence.
(A-B) Region of the inner ear in the posterior part of an intact half skull of a P5 *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ mouse.
(C-D) Removal of the skin and part of the skull allows better visualization of the region of the endolymphatic sac.
(E, F) Isolated inner ear with the endolymphatic sac still associated with conjunctive tissue.
(A, C, E) Images captured in incident light. (B, D, F) tdTomato fluorescence of the corresponding tissue. Scale bars: 2 mm (A-B), 1 mm (C-F).

Supplementary Figure 2. Comparison of the fluorescence of isolated endolymphatic sacs of *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ and *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$ littermate mice at E16.5 and P5.
tdTomato fluorescence greatly facilitates the recognition of the endolymphatic sac and duct.
(A-C) Microdissected endolymphatic sacs of E16.5 *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ (A) and *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$ littermate mice (B, C).
(D-F) Microdissected endolymphatic sacs of P5 *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$;Tg(ATP6V1B1-Cre)$^{1Rnel/Mn}$ (D) and *R26*$^{LSL\text{-}RCL\text{-}tdT/+}$ littermate mice (E, F).

(A, B, D, E) tdTomato fluorescence. (C, F) Incident light images corresponding to B and E. Scale bars: 500 μm.

DISCUSSION:

Many researchers have used paraffin-embedded or frozen sections for morphological studies of the endolymphatic sac. The disadvantage of sectioned preparations is that it can be difficult to fully observe the complicated cell-to-cell contacts because the size and shape of each epithelial cell in the endolymphatic sac is extremely variable, forming a wrinkled and pseudostratified cell layer. Whole mount immunostaining and z-stack imaging, described in this protocol, permit better visualization of the three-dimensional structure of the endolymphatic sac.

For successful microdissection, the critical point is to mentally visualize the entire outline of the endolymphatic sac, even if it is invisible in the wild type mouse. Observation of the fluorescent reporter shown in **Figure 3** and in **Supplementary Figure 1-2**, can be helpful for recognizing localization and size of the endolymphatic sac in mice.

While this whole-mount dissection method was used for immunostaining in the above protocol, this technique can also be used to harvest endolymphatic sac tissue for gene expression analysis, such as RT-qPCR, microarray expression, and even single-cell RNA-seq. The results of single-cell RNA-seq analysis using tissue prepared with this protocol have been previously reported[16]. For gene expression analysis of endolymphatic sac epithelial cells, it is preferable to collect isolated epithelium so that there is no contamination with other adjacent tissues. Although the embryonic endolymphatic sac can be separated from the adjacent connective tissues, it is much harder to do at later ages. For specimens from older mice, incubation with collagenase/dispase for 5 min at 37 °C is encouraged and facilitates the isolation of the endolymphatic sac epithelium.

One of the drawbacks of the whole-mount immunostaining described in this protocol is that the original three-dimensional structure may be affected by trauma during dissection or mounting of the preparation between glass slide and coverslip. Care is needed to avoid artefacts due to manipulation.

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DISCLOSURES:

The authors have nothing to disclose.

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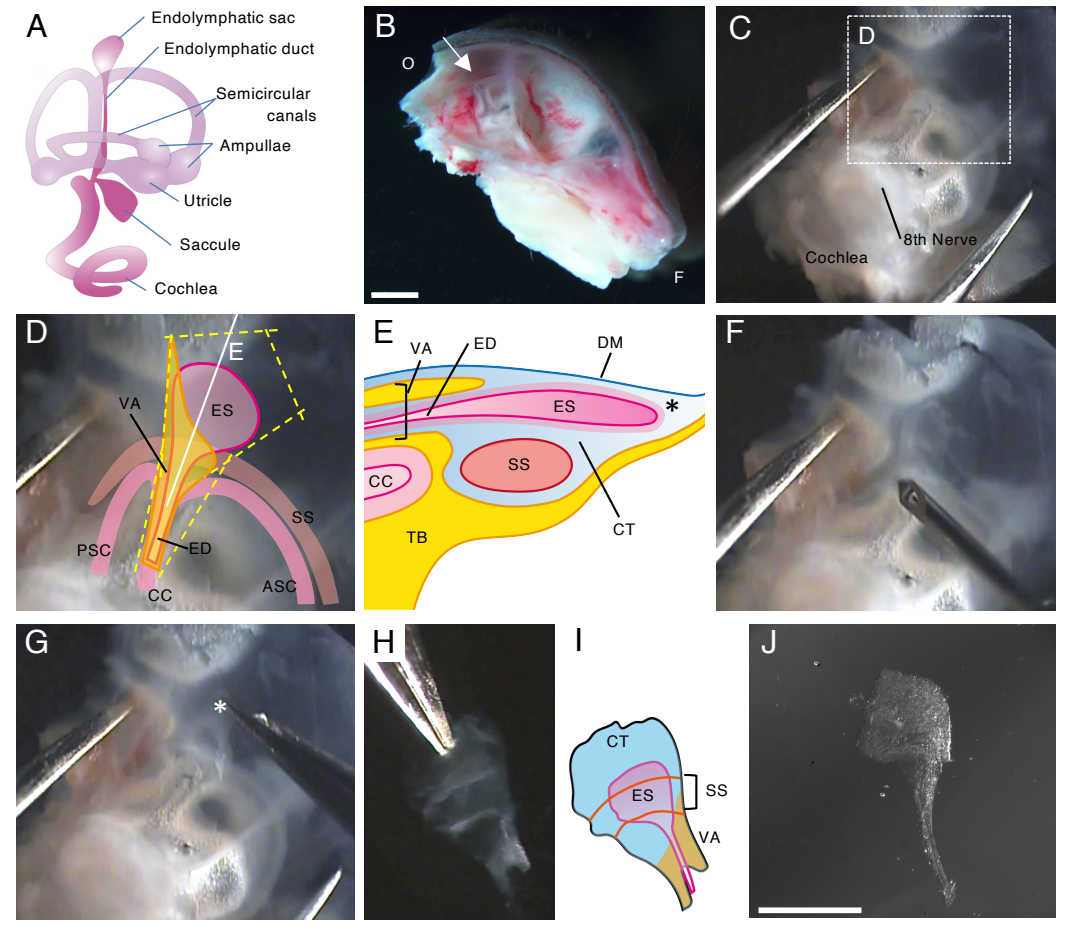
A
Endolymphatic sac
Endolymphatic duct
Semicircular canals
Ampullae
Utricle
Saccule
Cochlea
B
O
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D
8th Nerve
Cochlea
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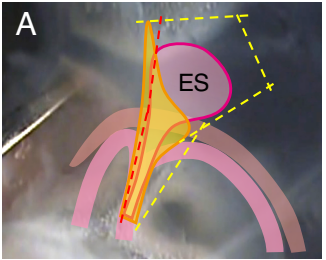
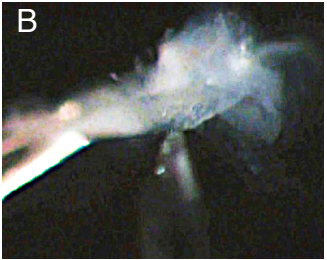

A
ES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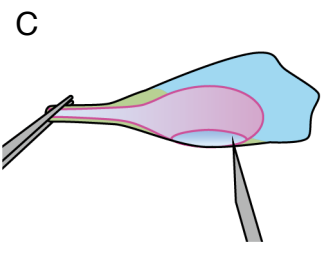
C


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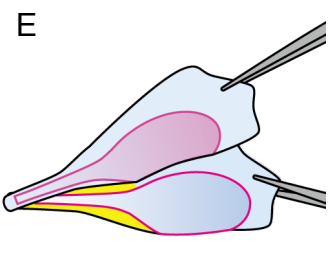
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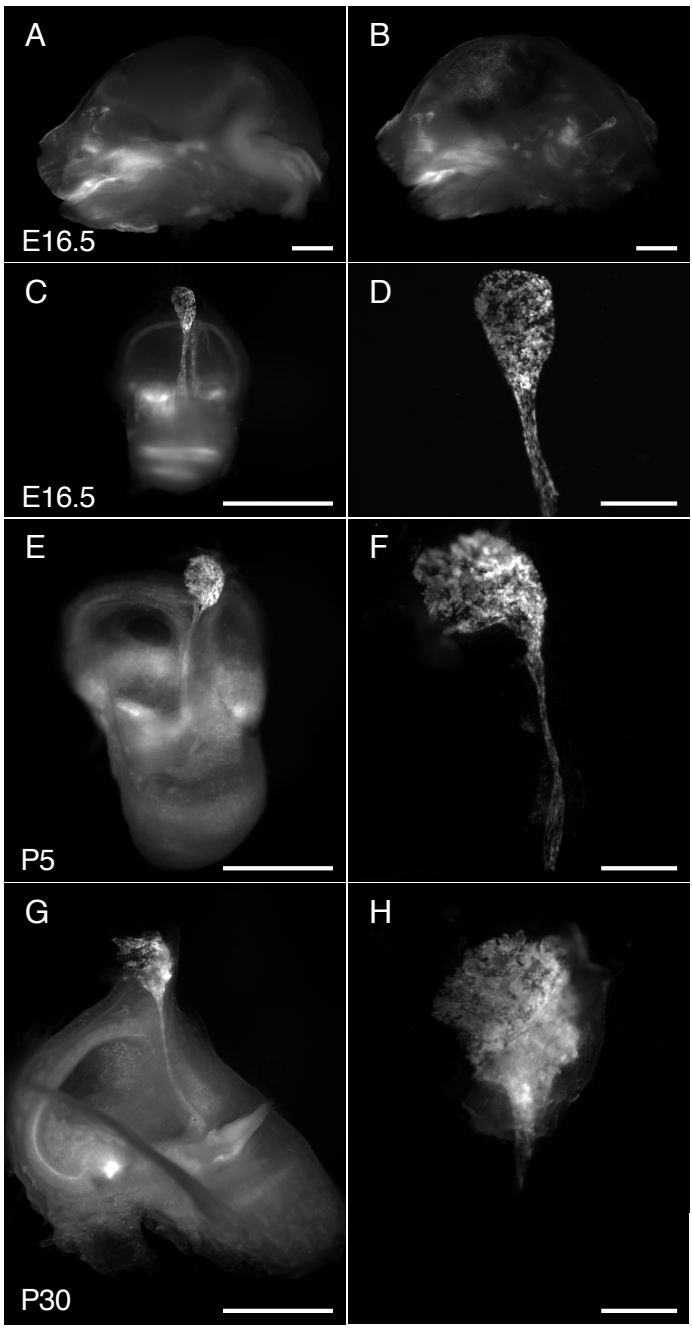
A
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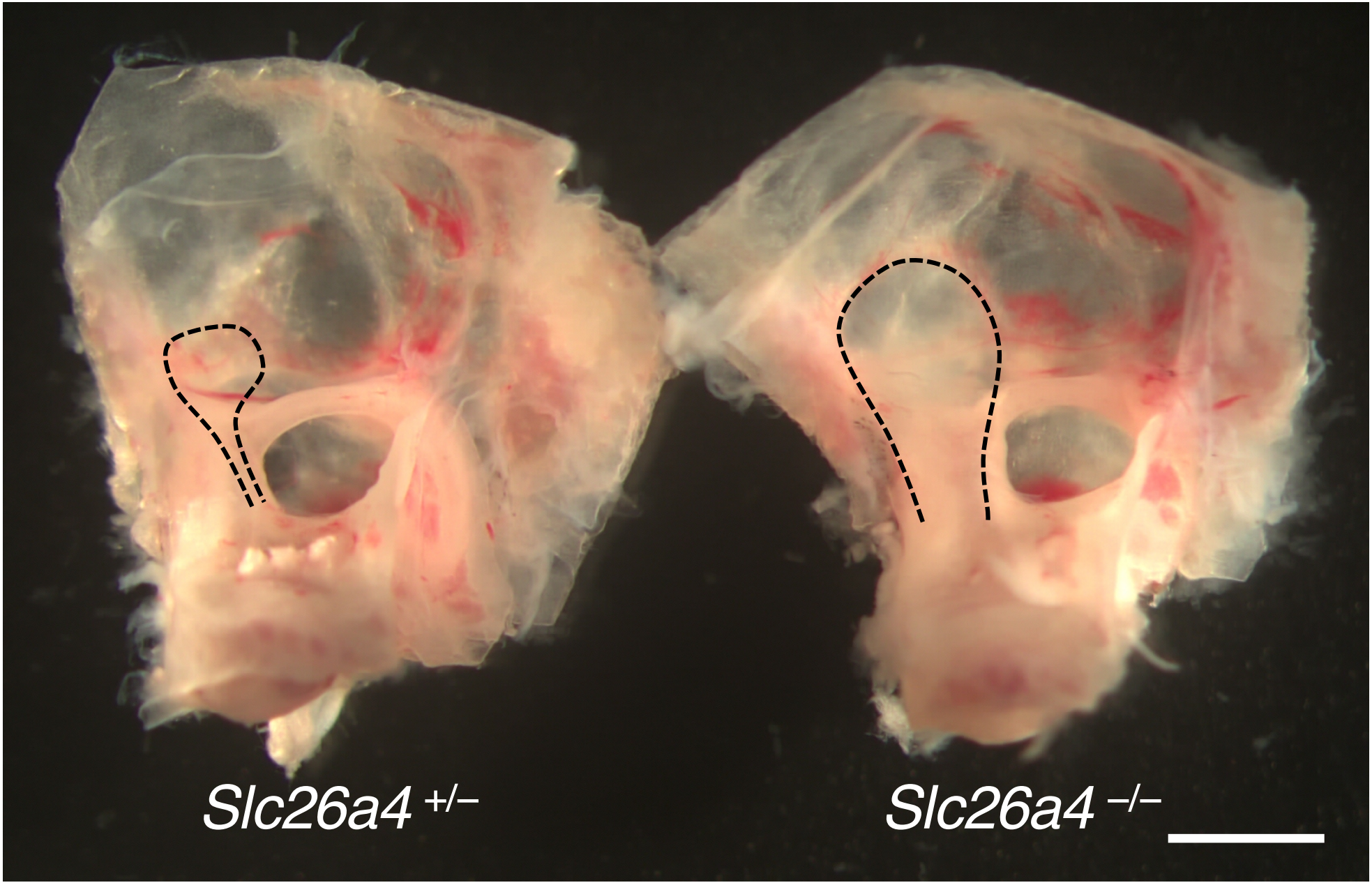

Slc26a4 +/–
Slc26a4 –/–

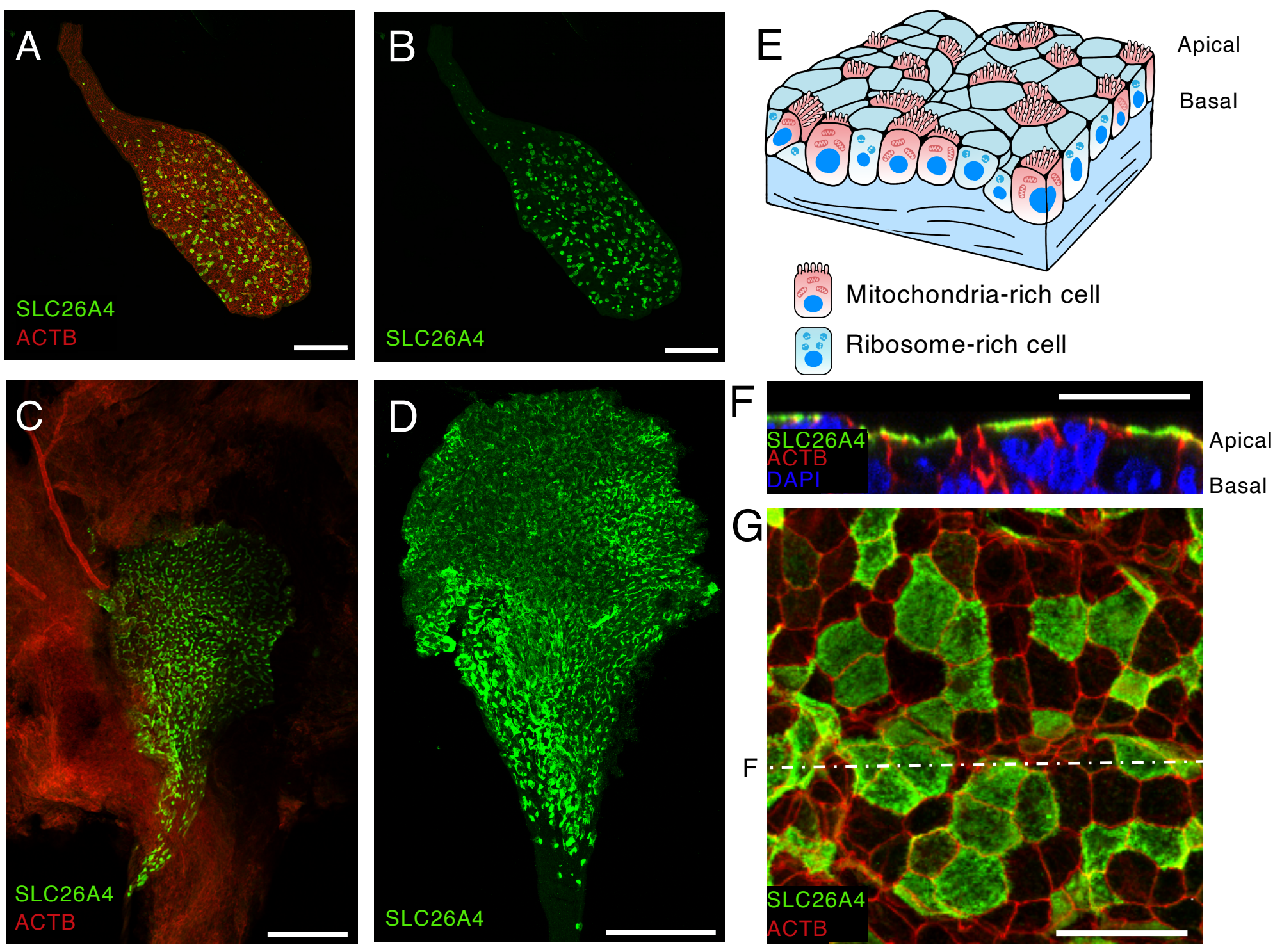

A
SLC26A4
ACTB
B
SLC26A4
E
Apical
Basal
Mitochondria-rich cell
Ribosome-rich cell
C
SLC26A4
ACTB
D
SLC26A4
F
SLC26A4
ACTB
DAPI
Apical
Basal
G
F
SLC26A4
ACTB

Table of Materials

Click here to access/download;Table of Materials;JoVE_HondaLee_Materials_02242021.xls

Name of Material/ Equipment	Company	Catalog Number
EDTA 0.5 M	Crystalgen	221-057
1 mL TUBERCULIN syringe with 27G x 13mm	BD	309623
9-well spot glass plate	Pyrex	13-748B
Alexa Fluor 555-conjugated phalloidin	ThermoFisher Scientific	A34055
Artery Scissors - Ball Tip	Fine Science Tools	14080-11
Bovine serum albumin	Sigma Aldrich	A3059
Camera to capture images in incident light	Leica	DFC 495
Camera to capture fluorescent images	Leica	DFC 7000 GT
Centrifuge tubes 15 mL	Corning	430053
Centrifuge tubes 50 mL	Corning	430829
Collagenase/Dispase	Roche	10269638001
Confocal microscope	Zeiss	LSM 880
Cover Glass	Corning	2940-223
Dumont #4 Forceps	Fine Science Tools	11241-30
Dumont #5 Forceps	Fine Science Tools	11251-20
Dumont #55 Forceps	Fine Science Tools	11255-20
Extra Fine Bonn Scissors	Fine Science Tools	14084-08
Straight; Sharp Points; 20mm Blade Length; 3 1/2" Overall Length	Roboz	RS-5880
Goat anti-rabbit Alexa Fluor 488-conjugated secondary antibody	ThermoFisher Scientific	A11034
MicroSlides Superfrost	VWR	48311-702
Orbital shaker, for example Mini Shaker	Daigger	980275
Paraformaldehyde (PFA) 16% Aqueous Solution	Electron Microscopy Sciences	15710
Phosphate-Buffered Saline (10X) pH 7.4	ThermoFisher Scientific	AM9624
ProLong Gold Antifade Mountant with DAPI	ThermoFisher Scientific	P36931
Rabbit anti-Pendrin antibodies	In-house	
Ring Forceps	Fine Science Tools	11106-09
Rocking shaker, for example GyroMini	Labnet	S0500
Stereomicroscope equipped with a PlanApo 1.0x objec	Leica	

Stainless Steel Single Edge Blades, .009"	GEM Personna	62-0176
Tissue culture dish 60 mm	Falcon	353002
Transparent nail polish		
Triton X-100	ACROS Organics	32737-1000

Comments/Description

RRID:AB_2713943, PB826 (Choi *et al.,* 2011)

Dear Dr. Nam Nguyen,

Thank you for your interest in our work and for considering our manuscript, JoVE62375 "Dissection of the Endolymphatic Sac from Mice", for publication in JOVE. Please find below our responses, in red, addressing each of the editorial and peer review comments. We are uploading in parallel our manuscript with the requested changes with track changes highlighting all of the edits, and the edited videos, for your consideration. Please contact us if you have any additional questions or concerns.

With Best Wishes,

Dr. Keiji Honda and Dr. Isabelle Roux

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We carefully proofread the manuscript.

2. Please revise the following lines to avoid previously published work: 114-115, 250-257.

Lines 114-115 “mice were backcrossed for at least five generations onto a C57BL/6J background before being bred for experiments” now reads “mice previously backcrossed to strain C57BL/6J for at least five generations, were bred for experiments.”

Lines 250-257 now read “Schematic of the membranous labyrinth of a developed mouse inner ear (right). The location of the endolymphatic sac, and other structures of the cochlea and vestibula are indicated.”

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns were removed except in the Acknowledgement. Please let us know if this section needs to be modified as well.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: GyroMini, Labnet, Daigger, Invitrogen, Zeiss LSM 880, ProLong, etc.

All commercial language was removed, the information and references of products used are now only included in the Table of Materials.

5. Line 121-124: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. (e.g., How is the animal euthanized? What is the concentration of the euthanizing agent? How are the embryos harvested?)

This section now reads:

Euthanize experimental animal or time-pregnant dam using an approved institutional procedure, for example using C02 inhalation. Work under a fume hood if possible to avoid the spread of mouse allergens. After verifying the lack of response to painful stimuli such as paw and tail pinches, decapitate the animal. For time-pregnant dam, cervical dislocation can be used as an alternative to decapitation if their inner ears are not used for further experiments. Harvest the embryos by laparotomy[14] using artery scissors with a ball tip to protect the embryos during the process. Manipulate the embryos using ring forceps. Place the uterine horns in 4°C PBS on ice and isolate each embryo as previously described[15] before decapitating them.

Two references published in JoVE describing the procedures used were added:

14. Wang, L., Jiang, H. & Brigande, J. V. Gene transfer to the developing mouse inner ear by in vivo electroporation. *J Vis Exp.* (64), (2012).
15. Currle, D. S., Hu, J. S., Kolski-Andreaco, A. & Monuki, E. S. Culture of mouse neural stem cell precursors. *J Vis Exp.* (2), 152, (2007).

6. Line 126-131: The Protocol should contain only action items that direct the reader to do something. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The text was modified accordingly.

7. Line 135: Please specify if there is any specific volume of PFA to be used.

A volume of 10 mL was indicated.

8. Line 146-147: Please specify if there is any specific volume of PBS used for washing.

A volume of 10 mL was indicated.

9. Line 191: Please use standard abbreviation for length units. Examples: 10 μL. 50 mL.

This was corrected all along the manuscript.

10. Line 191-192: Please specify the volume of permeabilizing and blocking solution used.

This information was added.

11. Line 195-196: Please include the volume of the antibodies used.

This information was added.

12. Line 213: Please add more details to the imaging steps. At what magnification was the imaging performed? Were there any specific system settings required to image the tissue? Please cite a reference if necessary.

This section now reads:
3.6. Image using a confocal microscope. Endolymphatic sac preparations are first observed with a 10x objective to have a global view of the sac labeling (Figure 5 A-D). A 63x objective is then used to gain a detailed view of the distribution of the protein of interest in mitochondria and ribosomal rich cells which compose the endolymphatic sac (Figure 5 F-G).

Settings will depend on the fluorophore used.

13. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be

visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have edited the video including the main steps of the Protocol for your consideration.

These steps have also been highlighted in yellow in the Protocol.

Please let us know if any additional information is needed.

14. Please do not use the &-sign or the word "and" when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

This was corrected.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The figure we would like to include was published in eLife. As eLife applies the Creative Commons license to their articles, there is no need of permission. We included in the legend of Figure 1: "This figure has been modified from Honda *et al.*[16]." We have also asked the permission to use this figure to Dr. Wangemann, who made this figure initially, and approved its use for the present publication.

16. Please sort the Table of Materials in alphabetical order.

This was done. Some information and references of products used were added in the Table of Materials as you suggested their removal from the main text.

For the video, please mark-up or annotate the video to help our editor navigate the file. You can also further break up the video into individual files of whatever parts they'd like to use for the particular sections of the video. You can upload the video sections here:
https://www.dropbox.com/request/KwzdlehauQxwKltpo6Ji?oref=e

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The Authors describe a method to dissect the endolymphatic sac in a mice. The paper is well written and concise. This article could be of some interest to the readers.

We thank the reviewer for his/her/their interest in this work and in this publication.

Minor Concerns:

Some of the figures have not clear labels

Figure 1: the exclamation points in E and G have been replaced by asterisks.

We separated the right part of panel H in its own panel now referred to as I.

The legend now reads:

"(G) The preparation is carefully removed from the temporal bone by holding the tissue at the position shown by the asterisk (*, also shown in panel E) and peeling it up.
(H) An isolated whole endolymphatic sac with surrounding tissues.
(I) Schematic version of this isolated endolymphatic sac with surrounding tissues.
(J) Isolated endolymphatic sac without surrounding tissues.
Scale bars: 2 mm (B), 1 mm (J)."

Figure 5: To clarify the presence of the panels F and G the legend now reads:

"(F-G) High-magnification maximum intensity projections images of endolymphatic sac epithelium at P5, after the endolymphatic sac was opened and labeled with an anti-SLC26A4 antibody (green) and phalloidin (ACTB, red). A representative image at the apical membrane level is shown in G, and a reconstructed cross-section from z-stack at the level of the punctate white line is shown in F. The nucleus of the cells is labeled with DAPI (blue)."

Reviewer #2:

The article has major flaws, that in my opinion, are basic flaws with the design of the study and hence cannot be rectified.

1. The aims and objectives are poorly and randomly defined
2. The introduction does not explain the rationale of the study and its potential utility
3. The methods though appearing to be an outcome of hard work, do not describe the settings, the outcome measures or any control group
4. The results do not contain any reference to the outcome of the steps detailed in

methodology. Completely new ideas and techniques are discussed in no particular order
5. The discussion is irrelevant.

We appreciate this reviewer's comments but they do not seem to understand the purpose of a JoVE manuscript. We do not think they are relevant to our manuscript. Please let us know if you would like us to respond in detail to these criticisms.

Reviewer #3:
Manuscript Summary:
In this manuscript, Honda et al. describe how to dissect the endolymphatic sac from the inner ear of the mouse. Authors also used a transgenic mouse with a fluorescent reporter so the readers can easily recognize its structure and anatomy. Figures are concise and well enough; the attached video demonstration is nice and beautiful. The reviewer consider it to be appropriate for publication in JoVE after following revision.

We thank the reviewer for his/her/their interest in this work and in this publication.

Major revision:
I am afraid the anatomy and each procedure in the video is still difficult to understand for the beginners even though the figures are well instructive. The reviewer strongly recommend authors to add voice narration explaining procedure held in each step.

We have edited the video so that each step of the dissection is now indicated in the video, some pauses have also been included to help the viewer. We also overlaid some schematics to highlight the different anatomical structures of the inner ear at different steps of the dissection to help the experimenter orient his/her/themselves and facilitate their reproduction of the procedure.

Reviewer #4:
Manuscript Summary:
This paper describes the methods of dissecting the endolymphatic sac from the inner ear of the mouse at different ages. The paper is well written and should facilitate further characterization of physiology and pathology of endolymphatic sac diseases.

We thank the reviewer for his/her/their interest in this work and in this publication.

This paper only requires minor revisions or clarification before being accepted.

Minor Concerns:
1. Line 240 to 243: The definition of the apical and basal surface of the endolymphatic sac is not clear and should be further clarified.

This paragraph has been modified to clarify the significance of apical *vs* basal surface:

It now reads:

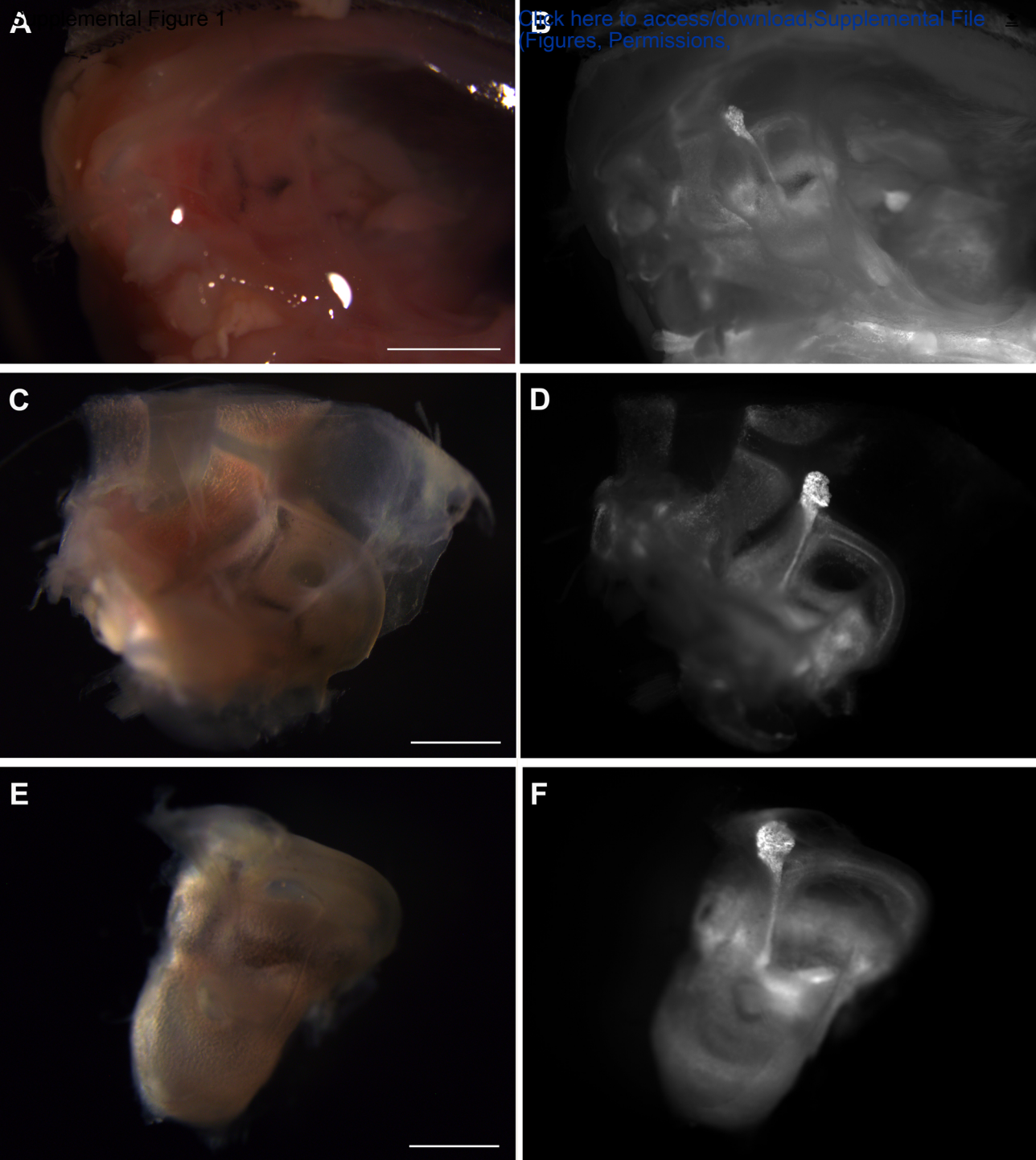
The endolymphatic sac is composed of a single layer of epithelium folded in a pouch-shaped structure, containing in its lumen endolymph. This epithelium consists of two cell types, mitochondria-rich cells and ribosome-rich cells (Figure 5E). A whole mounted endolymphatic sac will be flattened as endolymph is no more present and will show as two layers of epithelium intertwined, making it difficult to study subcellular localization of proteins expressed in this epithelium. By opening the endolymphatic sac, the single layer epithelium can be readily visualized and the relative distribution of the proteins of interest as compared to the endolymphatic sac lumen can be studied more easily. A potential apical (luminal, where endolymph would be) *versus* basal enrichment of these proteins can be more easily identified. As an example, SLC26A4 is enriched on the apical side of mitochondria-rich cells (Figure 5F).

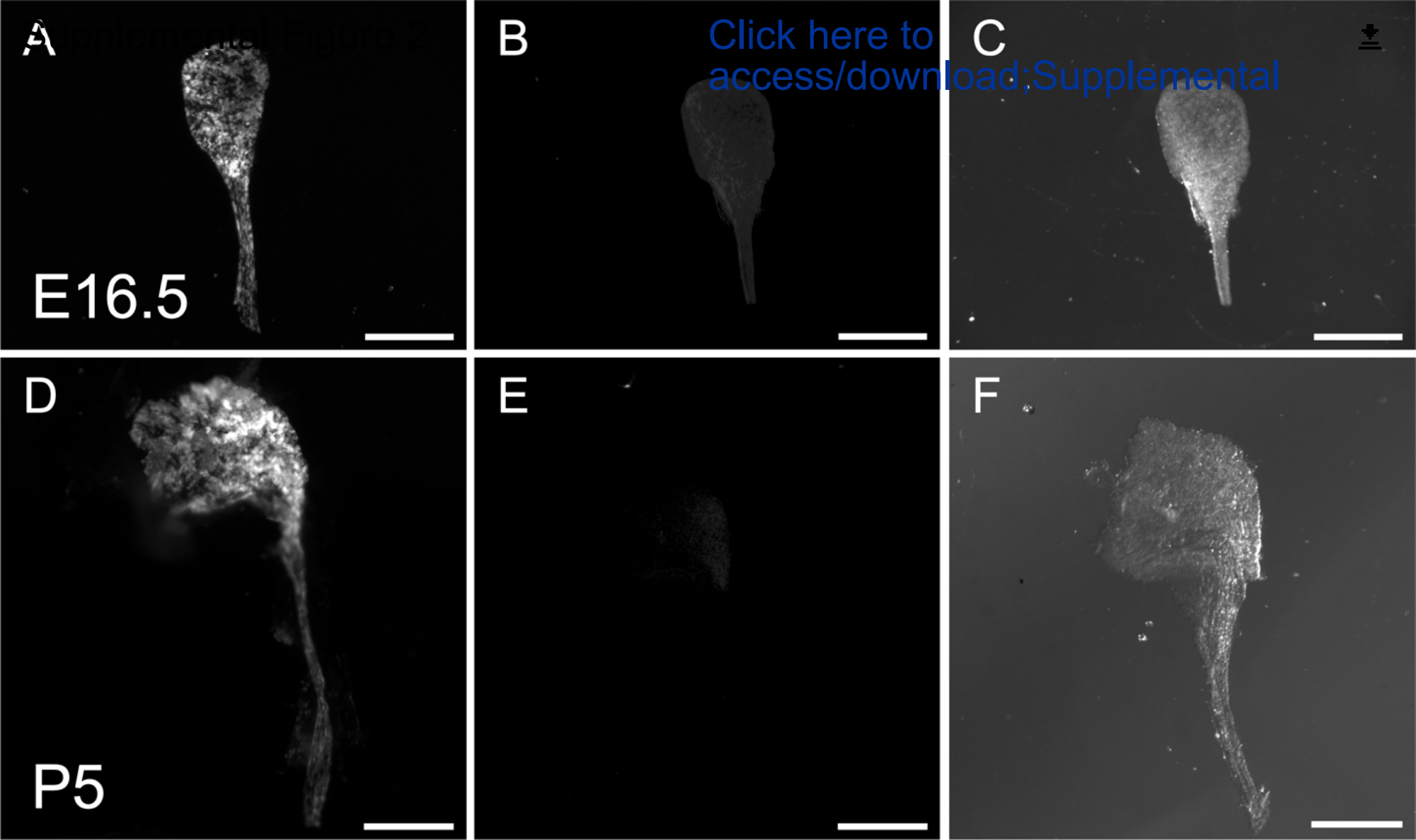
2. Line 242 and Fig 5F: "SLC26A4 is enriched on the apical side of mitochondria-rich cells" is not clear to me. How do you identify the "mitochondria-rich cells" in the figure? Can you show them in the figures from both apical and basal surface?

Previous work has shown that SLC26A4 is enriched in mitochondria-rich cells (Honda *et al.* 2017). Panel 5F shows a reconstructed cross section view at the level of the line indicated in panel G. This panel F gives access to a view of both the apical and basal surface of the endolymphatic sac epithelium. SLC26A4 labeling is only detected in the apical surface of the epithelium. To clarify this, we have added “apical” and “basal” on panel F.

3. Figure 5F and 5G: the authors showed beautiful SLC26A4-positive cells by immunofluorescent imaging. However, the details information of "ACTB" in not described. I suppose it to be beta-actin. The authors should show the detail information about this marker and the antibody origin.

Thank you for pointing this out. We have now clarified these points in the figure legend which now reads: “ High-magnification maximum intensity projections images of endolymphatic sac epithelium at P5, after the endolymphatic sac was opened and labeled with an anti-SLC26A4 antibody (green) and Phalloidin (ACTB, red).”
The detail information and origin of Alexa 555-Phalloidin are available in the excel file attached to the manuscript as recommended by the editor.




A
Supplemental Figure 2
B
Click here to access/download;Supplemental
C
E16.5
D
E
F
P5