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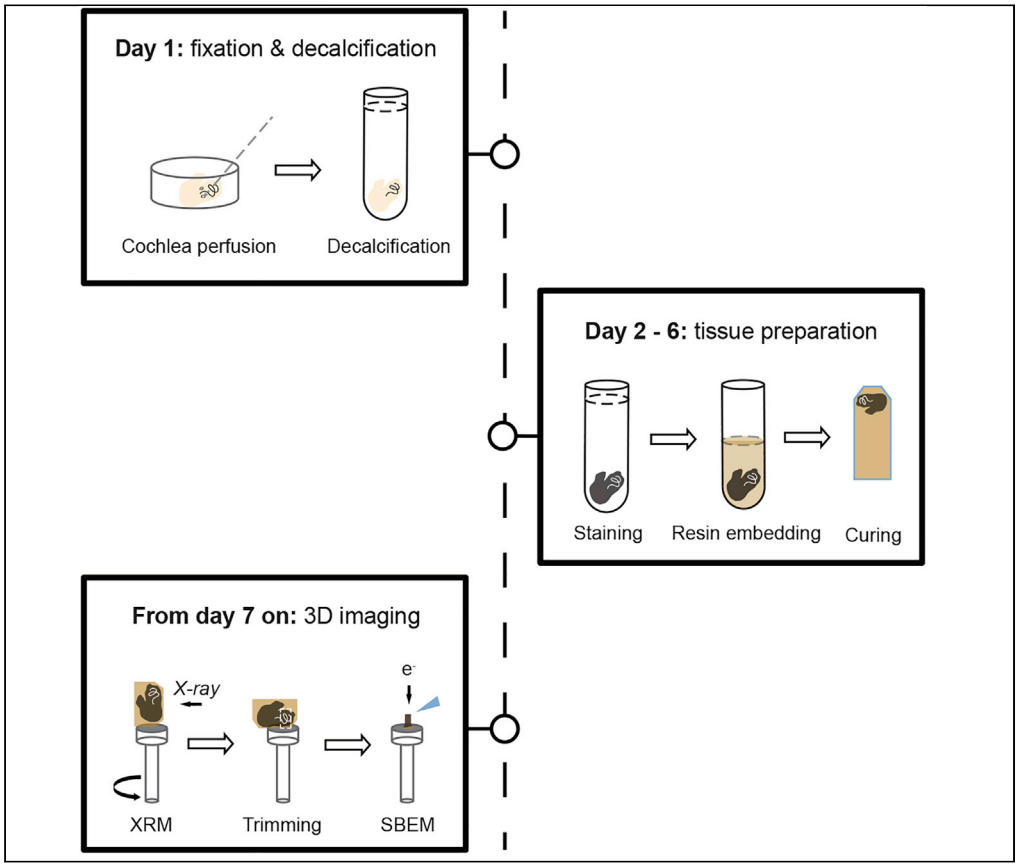
Protocol

Large-scale 3D imaging of mouse cochlea using serial block-face scanning electron microscopy



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Highlights

En bloc staining and resin embedding of whole mouse cochlea

3D imaging of cochlea at cellular resolution using compact X-ray microscopy

Large-scale volume electron microscopy imaging of cochlea

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Large-scale 3D imaging of mouse cochlea using serial block-face scanning electron microscopy

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SUMMARY

This protocol describes how to prepare intact mouse cochleae for serial block-face scanning electron microscopy (SBEM). The detailed workflow includes cochlea fixation, *en bloc* staining, resin embedding, X-ray microscopy-guided trimming and SBEM data acquisition. This protocol allows large-scale, nanometer-resolution three-dimensional imaging of subcellular structures in a targeted tonotopic range of the cochlea and enables fast volumetric scan at submicron resolution using a compact X-ray microscope.

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

BEFORE YOU BEGIN

As structural investigations using three-dimensional electron microscopy (EM) are oftentimes labor-intensive and time-consuming, it is highly recommended to document as much information as possible about the functional aspects of the test subject beforehand.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Chloral hydrate	Sigma-Aldrich	Cat# 23100
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Cat# EDS
Glutaraldehyde solution (70%)	Sigma-Aldrich	Cat# G7776
L-aspartic acid	Sigma-Aldrich	Cat# A8949
Lead nitrate	Emsdiasum	Cat# 17900
Osmium tetroxide (OsO_4, 4% aqueous solution)	Ted Pella, Inc.	Cat# 18465
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat# P6148
Potassium hydroxide solution (KOH, 8.0M)	Sigma-Aldrich	Cat# P4494
Potassium hexacyanoferrate ($K_4Fe(CN)_6$)	Sigma-Aldrich	Cat# P3289
Sodium cacodylate trihydrate	Sigma-Aldrich	Cat# C0250
Sodium hydroxide solution (NaOH, 5.0M)	Sigma-Aldrich	Cat# S8263
Spurr Low Viscosity Embedding Kit	Sigma-Aldrich	Cat# EM0300
Thiocarbohydrazide (TCH)	Sigma-Aldrich	Cat# 223220
Dual-component epoxy adhesive	UHU	Cat# 63251

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: organisms/strains		
Mouse	N/A	N/A
Software and algorithms		
Dragonfly	Objective Research Systems Inc.	https://www.dragonflysoft.cn
Fiji/ImageJ	(Schindelin et al., 2012)	https://imagej.net/Fiji
Other		
Field-emission scanning electron microscope	Carl Zeiss Microscopy	GeminiSEM 300
X-ray microscope	Carl Zeiss Microscopy	Xradia 520 Versa
3View2XP in-chamber microtome	Gatan, Inc.	N/A
OnPoint backscattered electron (BSE) detector	Gatan, Inc.	N/A
208C High Vacuum Turbo Carbon Coater	Ted Pella, Inc.	Cat# 9620-220
Gold sputter coater	Leica	ACE600
Ultramicrotome	Leica	EM UC7
Trimmer	Leica	TRIM2
Oven	Shanghai Yiheng Instruments Co., Ltd.	Cat# DHG-9015A
Rotator	Crystal Technology & Industries, Inc	Cat# TR-05UA
Perfusion pump	Harvard Apparatus	Cat# 70-3007
Diamond ultramicrotome knife	DiATOME	Cat# T-2178
Diamond trimming knife	Anton Meyer & Co. Ltd	Cat# 227172
Folded filter paper	Whatman	Cat# 10311647
Aluminum metal rivet	Gatan, Inc.	3VMRS12

Note: We have tested staining using TCH from an alternative supplier (ScienceServices, Cat# 223220, https://www.scienceservices.de/thiocarbonohydrazid-tch-hochste-reinheit-5g.html). The reagent may appear in different colours. In our hands, white and light pink crystalline work best in terms of minimizing charging during SEM scanning. One can quickly test the effectiveness of the TCH solution by adding one drop of 2% OsO_4 solution, which should immediately turn the mixture into a brown colour.

MATERIALS AND EQUIPMENT

- 0.3M cacodylate buffer: Dissolve 32.1 g sodium cacodylate trihydrate in 500 mL Millipore water. When the solid is fully dissolved, adjust pH to 7.4 with 10% hydrochloric acid. Stored at 4°C before use.

Note: The cacodylate buffer can be stored at 4°C for months. Filter before use.

0.3M cacodylate buffer	32.1 g sodium cacodylate trihydrate, fill up to 500 mL with Millipore water. Adjust pH to 7.4 with 10% hydrochloric acid.

⚠ CRITICAL: Cacodylate contains arsenic, which is highly toxic. It may cause poisoning and even cancer if swallowed or inhaled. It should be handled in a fume hood while wearing protective gloves and a lab coat.

- Fixative: Dissolve 6 g paraformaldehyde (PFA) in 150 mL warm water (50°C–60°C) by stirring in a fume hood. After the PFA powder is almost dissolved (to speed up the dissolving process, few drops of NaOH solution may be added to the solution), cool the clear solution down to 20°C–

24°C and filter it with folded filter paper. Add 80 mL 0.3M cacodylate buffer and 10.7 mL 70% glutaraldehyde, fill up with Millipore water to 300 mL.

Note: Prepare the fixative freshly (less than 2 h before use) and store at 4°C.

Reagent	Final Concentration	Amount
PFA	2%	6 g
70% glutaraldehyde	2.5%	10.7 mL
0.3M cacodylate buffer	0.08M	80 mL
Millipore water	n/a	209.3 mL
Total		**300 mL**

⚠ CRITICAL: PFA, glutaraldehyde and cacodylate should be handled in a fume hood while wearing protective gloves and a lab coat.

- 5% ethylenediaminetetraacetic acid (EDTA) solution: Add 5 g EDTA and 26.7 mL 0.3M cacodylate buffer to 50 mL filtered PFA solution (4%). Add slowly 5.0M NaOH solution (~10 mL) under stirring. When the EDTA solid is fully dissolved, filter the clear solution with folded filter paper. Add 3.6 mL 70% glutaraldehyde and fill with Millipore water up to 100 mL.

Note: Prepare the EDTA solution about 2 h in advance due to slow dissolving process.

Note: As the EDTA solution is mixed with PFA and glutaraldehyde, it is recommended to prepare the solution just before use for a good post-fixation.

Note: Prepare the EDTA solution freshly (2 h in advance) and store at 4°C before use.

Reagent	Final Concentration	Amount
4% PFA solution	2%	50 mL
EDTA	5%	5 g
70% glutaraldehyde	2.5%	3.6 mL
0.3M cacodylate buffer	0.08M	26.7 mL
5.0M NaOH	0.5M	~ 10 mL
Millipore water	n/a	~ 9.7 mL
Total		**100 mL**

⚠ CRITICAL: NaOH solution is corrosive and should be handled in a fume hood while wearing protective gloves and a lab coat.

- 2% buffered osmium tetroxide (OsO_4): 1:1 mix 4% OsO_4 aqueous solution with 0.3M cacodylate buffer.

Note: Prepare the OsO_4 solution freshly. Store in parafilm-sealed falcon tube for less than 6 h.

2% OsO_4	1:1 mix 4% OsO_4 aqueous solution with 0.3M cacodylate buffer

⚠ CRITICAL: OsO_4 is toxic and should be handled in a fume hood while wearing protective gloves and a lab coat.

- 2.5% buffered ferrocyanide solution: Dissolve 1.25 g potassium hexacyanoferrate ($K_4Fe(CN)_6$) in 25 mL 0.3M cacodylate buffer and 25 mL Millipore water. Filter with folded filter paper before use.

  Note: Prepare the ferrocyanide solution freshly. Store it in parafilm-sealed, light-protective falcon tube for less than 6 h before use.

Reagent	Final Concentration	Amount
$K_4Fe(CN)_6$	2.5%	1.25 g
0.3M cacodylate buffer	0.15M	25 mL
Millipore water	n/a	25 mL
Total		**50 mL**

- 1% thiocarbohydrazide (TCH): Dissolve 0.5 g TCH in 50 mL Millipore water by stirring at 20°C–24°C for about 2 h. Filter with folded filter paper before use.

  Note: TCH is sensitive to light and air. Fill up the 50 mL falcon tube with solution to minimize contact with air. Use light-protective falcon tube or wrap the tube with aluminum foil during mixing.

  Note: TCH dissolves slowly in water with a limited solubility, therefore prepare the solution in advance (about 2 h before use). Store in parafilm-sealed falcon tube in darkness only for a short period (less than 2 h).

1% TCH	0.5 g TCH, fill up to 50 mL with Millipore water

⚠ CRITICAL: TCH is toxic and should be handled in a fume hood while wearing protective gloves and a lab coat.

- L-aspartate buffer: Dissolve 2 g L-aspartic acid in 500 mL Millipore water. Adjust pH to 3.8–4.0 with 8.0M potassium hydroxide (KOH) solution.

  Note: The L-aspartate buffer can be stored at 4°C for months and filter with folded filter paper before use.

L-aspartate buffer	2 g L-aspartic acid, fill up to 500 mL with Millipore water. Adjust pH to 3.8–4.0 with 8.0M potassium hydroxide (KOH) solution

- Lead aspartate solution: Dissolve 0.165 g lead nitrate in 25 mL L-aspartate buffer. Adjust pH to 5.0 with 8.0M KOH.

  Note: Store in parafilm-sealed light-protective Eppendorf tube or in darkness to avoid air and light only for a short period (less than 1 h).

Lead aspartate solution	0.165 g Lead nitrate, fill up to 25 mL with L-aspartate buffer. Adjust pH to 5.0 with 8.0M potassium hydroxide (KOH) solution

Note: Prepare the lead aspartate solution just before use.

Note: Adjust the pH precisely.

⚠ CRITICAL: Lead nitrate is a white crystalline. It causes serious eye damage and should be handled in a fume hood while wearing protective gloves and a lab coat.

- Spurr resin: Weight 4.1 g ERL-4221 (VCH substitute, the original monomer is no longer available from the supplier due to high toxiticity), 0.95 g DER-736 and 5.9 g NSA with a 50 mL falcon tube. Gently mix the liquid until homogenous appearance in color. Add 1% DMAE to the mixture and mix thoroughly again.

Note: Prepare freshly.

Reagent	Final Concentration	Amount
ERL 4221	37.1%	4.1 g
DER-736	8.6%	0.95 g
NSA	53.4%	5.9 g
DMAE	0.9%	113 μL
Total		10 mL

Note: It is recommended to use freshly prepared resin. Handle gently to avoid inducing air bubbles during mixing.

⚠ CRITICAL: Spurr resin is a hydrophobic resin with a low viscosity, facilitating its penetration into tissues and dense structures. It is toxic and may damage the respiratory system. Resin should be handled in a fume hood while wearing protective gloves and a lab coat.

STEP-BY-STEP METHOD DETAILS

In this protocol, we describe all steps from cochlea fixation, *en bloc* EM staining, resin embedding, targeted sample trimming to SBEM data acquisition (Figure 1).

Fixation and decalcification of mouse cochlea

Timing: 1 day

1. Fixative preparation
   a. Prepare the fixative and the EDTA solution. Cool the solutions on ice before use.

⚠ CRITICAL: PFA, glutaraldehyde and cacodylate buffer should be handled in a fume hood while wearing protective gloves and a lab coat.

2. Cochlea perfusion
   a. Anesthetize the animal by intraperitoneal injection of 5% chloral hydrate (500 mg/kg body weight).
   b. Dissect the auditory bulla from both sides after decapitation.
   c. Immerse the tissue in ice-cold cacodylate buffer (0.15M, pH 7.4).
   d. Open both round and oval windows with fine-tip forceps under a dissecting microscope.
   e. Perfuse the cochlea with 1 mL ice-cold fixative by a flat-headed needle (23 G) through the round and oval windows at a constant flow rate (800 μL/min) using a perfusion pump. Repeat three times (Figure 2).

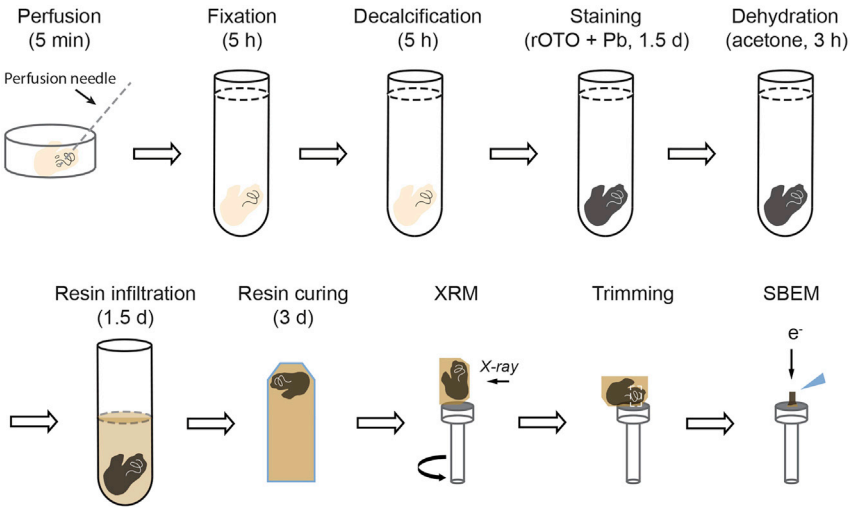

Figure 1. Schematic illustration of the workflow of sample preparation for SBEM imaging

The procedure includes cochlea perfusion, fixation, decalcification, EM staining, dehydration, sample embedding and trimming.

f. Remove surrounding muscle tissue with dissecting tweezer (optional).
g. Transfer the perfused cochlea into 5 mL Eppendorf tubes filled with ice-cold fixative.
h. Store at 4°C for 5 h.

⚠ CRITICAL: Perfusion should be done in a fume hood while wearing protective gloves and a lab coat.

⚠ CRITICAL: Handle the tissue carefully and always keep it immersed in ice-cold solution for optimal ultrastructure preservation.

Note: Don't cut apart the cochlea from the auditory bulla, which contains landmarks for determining the cochlea orientation.

Note: This protocol is dedicated for whole-cochlea-wide preservation of nerve structures, thereby a relative high perfusion pressue is used to ensure fast and homogenous fixation, which however may cause damage of fine filamentous structures at the hair cell tips.

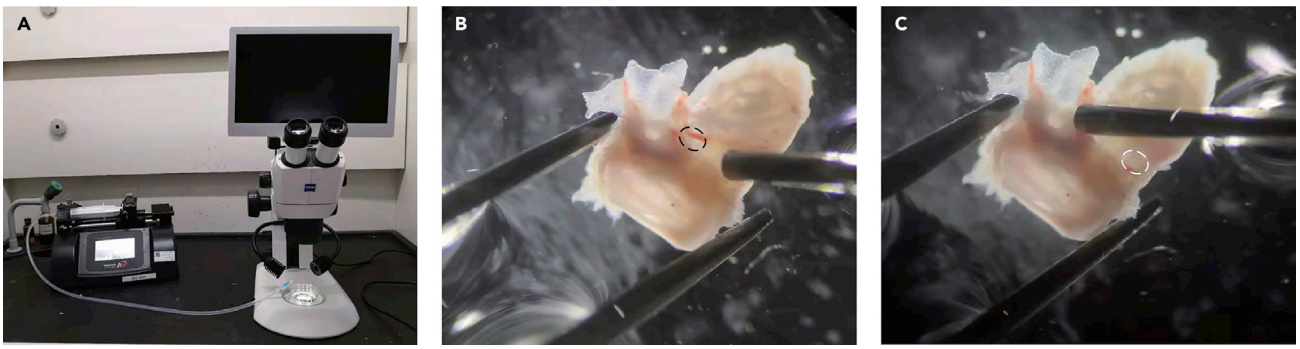

Figure 2. Cochlea perfusion

(A) Setups used for cochlea perfusion in a fume hood, including a perfusion pump and a dissecting microscope.
(B and C) Perfusion ice-cold fixative at a constant flow rate (800 μL/min) through oval (B) and round window (C) with a flat-headed needle.

3. Decalcification
   a. Transfer the sample to a 5 mL Eppendorf tube filled with the 5% EDTA solution.
   b. Keep it immersed in the EDTA solution. Store at 4°C for another 5 h.

   Note: In our experience, short decalcification duration leads to inhomogeneous staining/embedding due to poor tissue permeability for stain and resin, whereas prolonged incubation causes severe membrane extraction.

   Note: Consider replacing EDTA solution one or two times during the decalcification when incomplete calcification is noticed.

   Pause point: The fixed and decalcified samples can be stored in 0.15M cacodylate buffer at 4°C up to 2 days.

En bloc staining of cochlea for SBEM

Timing: 1.5 days

In this step, we describe the optimized staining protocol which is slightly modified from the previously reported procedure for preparing large-size brain tissue (Hua et al., 2015).

4. Wash the samples twice with 0.15M cacodylate buffer for 30 min each.
5. Immerse in 2% buffered OsO_4 solution at 20°C–24°C for 2 h.
6. Transfer to 2.5% buffered ferrocyanide solution at 20°C–24°C for 2 h.
7. Transfer to 2% buffered OsO_4 solution at 20°C–24°C for 1.5 h.
8. Wash with 0.15M cacodylate buffer for 30 min and then with Millipore water for 30 min.
9. Incubate in 1% TCH solution at 20°C–24°C for 1 h.
10. Wash with Millipore water twice for 30 min each.
11. Incubate in 2% OsO_4 aqueous solution at 20°C–24°C for 2 h.
12. Wash with Millipore water twice for 30 min each.

    Pause point: The sample can be stored at 4°C up to 12 h.

13. Incubate in freshly prepared lead aspartate solution at 50°C for 2 h.
14. Wash twice at 20°C–24°C with Millipore water for 30 min each.

    Note: The staining of cochlea sample is carried out in 2 mL Eppendorf tubes filled with different staining solutions. Sample transfer between tubes via disposable Pasteur pipettes.

Dehydration and resin embedding of cochlea

Timing: 2 days, 3 days curing

15. Incubate the sample sequentially in a graded acetone-water mixture.
    a. 50%, 75%, 90% acetone at 4°C for 30 min each.
    b. Pure acetone three times at 20°C–24°C for 30 min each.

    Note: Pre-cool 50%, 75%, 90% acetone to 4°C before use.

16. Transfer the sample in a 2 mL Eppendorf tube containing 1:1 mixture of acetone and Spurr resin. Mix at 20°C–24°C for 6 h on a rotator (speed: 5 rpm) with the tube horizontally placed along the rotation axis.

17. Transfer to a tube containing 1:2 mixture of acetone and Spurr resin at 20°C–24°C on rotator (tilted 60 degree from the horizontal plane, speed: 5 rpm). Keep the tube open to allow slow evaporation of the solvent for about 12 h.
18. Transfer to a tube containing pure Spurr resin and incubate at 20°C–24°C for 8–12 h. Keep the tube closed.

Note: Use freshly prepared resin. Freeze and thaw cycles may introduce water into the resin.

19. Transfer the sample to embedding mold and fill up with resin.
20. Incubate in a pre-warmed oven at 60°C for 24 h.
21. Incubate in the oven at 70°C for 48–72 h.

Note: Please see the hues of staining solutions and resins used in this protocol (Figure 3).

Fast sample preview and inspection using X-ray microscopy

Timing: 1–2 days

In this section, we describe how to identify the region of interest based on X-ray microscopy (XRM) data.

22. Sample mounting and trimming for X-ray microscopy.
    a. Use a scalpel or razor blade to carefully cut off blank resin from the sample block. Mount the sample (upright along the conical center axis of cochlea) on an aluminum metal rivet (Gatan) with two-component epoxy adhesive (UHU) for at least 12 h (Figure 4A).
    b. Remove excess resin around the cochlea as much as possible by a trimmer (Leica, TRIM2) equipped with a diamond knife head to yield the best possible resolution and improve the imaging quality.

Note: The cured resin can be very brittle. Slow trimming with caution is recommended to avoid sample damage.

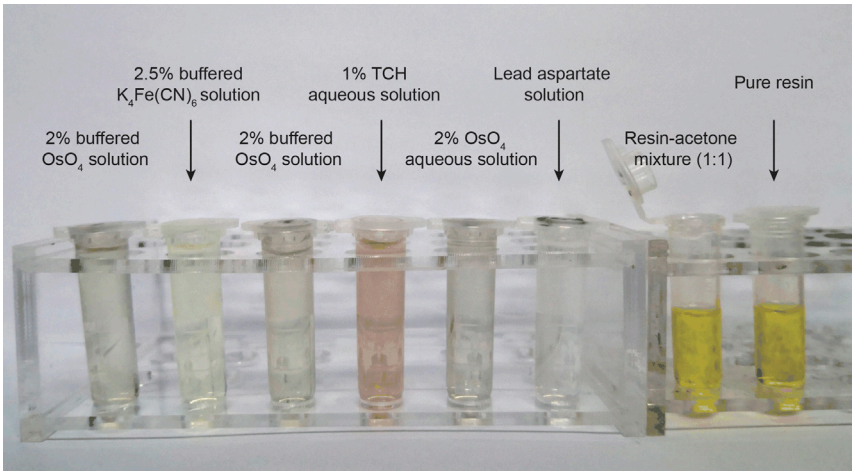

Figure 3. Hues of staining solution and resins used in this protocol
From left to right: 2% buffered OsO_4 solution, 2.5% buffered ferrocyanide solution, 2% buffered OsO_4 solution, 1% TCH solution, 2% OsO_4 aqueous solution, lead aspartate solution, 1:1 mixture of acetone and Spurr resin and pure Spurr resin.

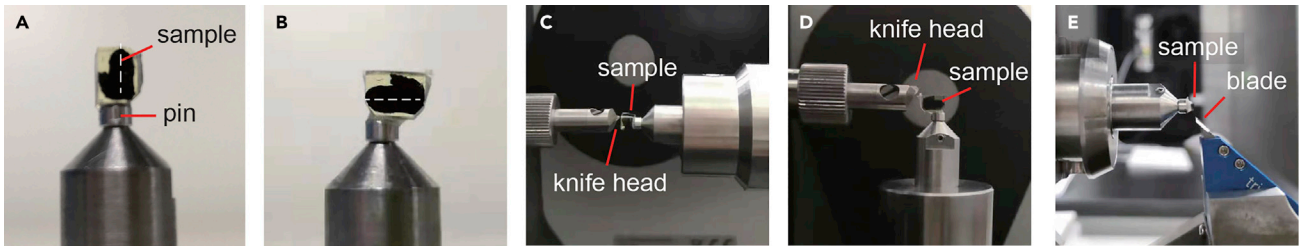

Figure 4. Sample mounting and trimming for XRM and SBEM imaging

(A) Resin-embedded cochlea sample was mounted vertically on the aluminum metal rivet for XRM imaging.

(B) The sample was remounted horizontally for SBEM imaging.

(C and D) Sample trimming from top surface (C) and sides (D) by a trimming machine with rotated specimen arm.

(E) Polishing of sample block-face using an ultramicrotome.

Note: This mounting direction is preferred for the ease of sample rotation in the X-ray microscope.

Note: Small sample dimension allows short distance between sample and the detector which is crucial to yield optimal resolution with the X-ray microscope.

23. Scan the sample with a high-resolution X-ray microscope (Zeiss, Xradia 520 Versa) at a pixel size of <4.5 μm, source voltage 60 kV, power 5 W and exposure time per radiograph of 1 sec. We recommend acquiring >1001 radiographs.
24. Import the reconstructed XRM dataset into 3D image processing software (XM3DViewer, Zeiss or Dragonfly, ORS Inc.) to visualize the 3D volumetric data (Videos S1 and S2)

25. Locate the region of interest using Dragonfly software:
    a. Annotate the whole extent of the basilar membrane using the built-in annotation tool.
    b. Label the locations of the frequency range of interest according to the reported place-frequency map of the mouse cochlea (Muller et al., 2005).
    c. Draw a bounding box around the locations of interest and measure the relative distances to the air-resin interfaces.

Note: The angle and distance measurements from the regions of interest to the air-resin interfaces is used to design a trimming plan in the subsequent sample preparation step.

Serial block-face electron microscopy

Timing: 2 days up to few months

26. Sample re-orientation: Use a razor blade to remove the sample from the aluminum metal rivet (after XRM scanning). Mount the cochlea sample (the conical center axis of cochlea in horizontal direction) on an aluminum metal rivet with two-component epoxy adhesive (UHU) for at least 12 h (Figure 4B).
27. Expose the tissue block at the selected region of interest by trimming from sample top surface using a trimmer (Leica, TRIM2) equipped with diamond knife head (Figure 4C).

28. Sample pre-scan using SEM (recommended)

Note: It is recommended at this step to evaluate the sample quality in terms of membrane contrast and ultrastructure preservation before investing time in sample trimming and initiating SBEM acquisition.

a. Coat the sample with 10-nm-thick carbon layer using a sputter coater (Ted Pella, 208C) and ground by connecting the sample surface with a carbon adhesive tape to the aluminum pin holder.
b. Scan the block-face by a SEM (GeminiSEM 300, Zeiss) equipped with a back-scattered electron detector.

Note: Block-face smoothing with an ultramicrotome is recommended for high-resolution imaging.

Note: Stitch via image processing tool (e.g., open-source stitching plug-in in Fiji using global optimization (Preibisch et al., 2009)), when both large field of view and high resolution are desired (Figure 5).

29. Trim down the sample block from the vertical sides by setting the specimen arm to 90 degree facing the sample milling direction in the trimmer (Figure 4D).

Note: The maximum sample width allowed at our SBEM setup is 1.2 mm.

30. Smooth the block-face by the ultramicrotome (Leica, EM UC7) equipped with a diamond trimming knife (Figure 4E).

Note: A block-face with minimum tilt from the horizontal plan can save cutting cycles with in-chamber ultramicrotome, and thereby prolong the SBEM kinfe lifetime. This is recommended, considering the high price and complexity of SBEM knife exchange.

31. Coat the sample with a 30-nm-thick gold layer using a sputter coater (Leica, EM ACE600).
32. Initiate SBEM imaging
    a. Center and secure the sample block in the in-chamber ultramicrotome (Gatan, 3View2XP) via adjusting and tightening screws on the aluminum pin holder.
    b. Elevate the sample holder to carefully advance the sample block-face toward the ultramicrotome knife by monitoring the knife shadow on the block-face. Cut with max. 80 nm step size, until the gold layer is completely removed from the block-face.
    c. Position the gas injection carbon needle of the focal charge compensation device (FCC, (Deerinck et al., 2018)) so that gas flow is directly pointing towards the block-face from a minimum distance (Figures 6A and 6B).
    d. Set SBEM imaging parameters and start data acquisition.

Note: Large single-tile image (20,000 × 15,000 pixels) of 11 nm pixel size and nominal cutting thickness of 40 nm was recently achieved (Hua et al., 2021; Wang et al., 2021). Incident

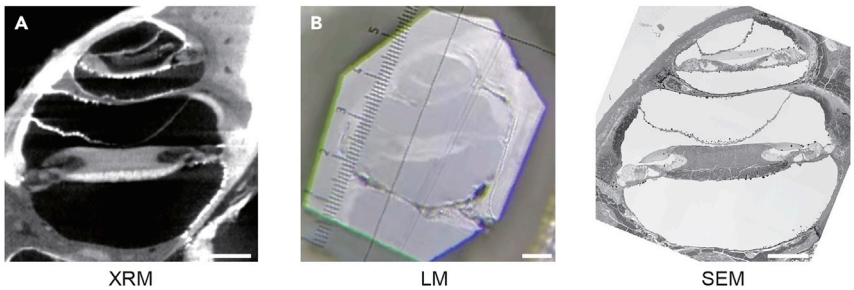

Figure 5. Correlative XRM and SBEM imaging
(A) Virtual cochlea coronal section of volumetric XRM data. Scale bar, 200 μm.
(B) The same region as in A under the dissecting microscope after trimming. Scale bar, 200 μm.
(C) Electron micrograph of the same region imaged at 30 × 30 nm^2 pixel size. Scale bars, 200 μm.

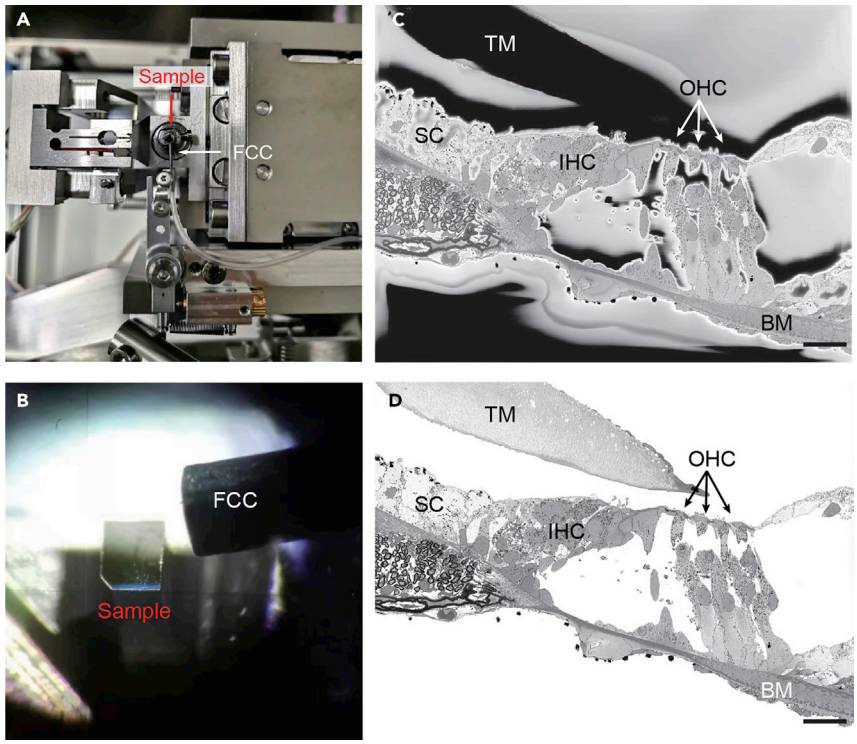

Figure 6. Effect of FCC on minimizing sample charging

(A) Arrangement of an in-chamber ultramicrotome with FCC device. Constant nitrogen gas flow is directed by a carbon needle (red arrow) towards the sample block-face.

(B) Magnified view of the FCC carbon needle above the sample block-face.

(C) Electron micrograph of the organ of Corti without FCC application. Note charge accumulation at pure resin area and structures like tectorial membrane (TM) and supporting cells (SCs).

(D) Electron micrograph from the same region as in C under the same imaging condition but with FCC. Scale bar, 20 μm.

beam energy 2 keV; dwell time 1 μs; FCC gas flow was set to 100% with a chamber pressure of ~ 2.8×10^{-3} mbar.

Note: The effect of FCC application minimizes charging artifacts (Figures 6C and 6D). An alternative approach to reduce charging artifacts is low-vaccum mode SBEM acquisition by inducing nitrogen or water-vapor. Compared to the FCC method, low-vacuum mode SBEM imaging, however, has lower signal detection efficiency.

Note: The FCC gas flow should be set just sufficient to cancel the charging artifacts, as gas injection reduces the signal-to-noise ratio of electron detection.

Note: Although the data acquisition of SBEM operates in a fully automated fashion, manual refocusing is required once a day on average based on our experience.

Note: Due to the spiral nature of cochlear basilar membrane, field of view adjustment is requried to follow the target structures during large image stack acquisition.

EXPECTED OUTCOMES

The workflow describes how to routinely generate large-scale 3D EM image dataset from targeted cochlea region (Figure 7). The *en bloc* staining protocol which was modified for intact mouse cochlea provides homogenous high membrane contrast and well-preserved ultrastructures, allowing quantifications of ribbon-type synapses, mitochondria, thin nerve fibers and etc. in a large context

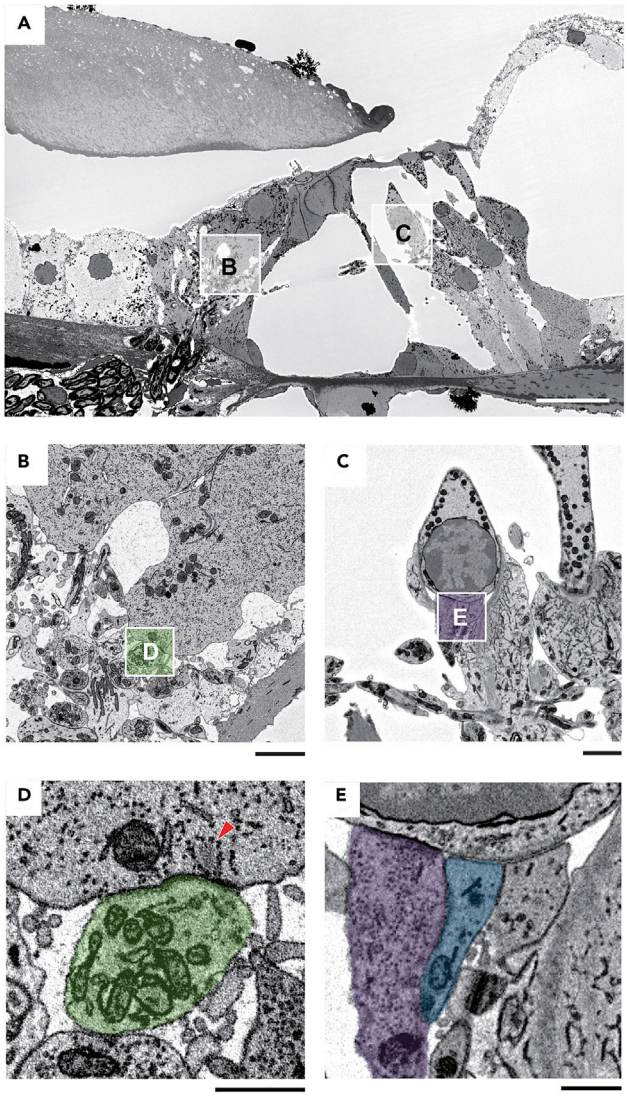

Figure 7. High-resolution SBEM imaging of mouse cochlea

(A) Field of view centered on the organ of Corti. Scale bar, 20 μm.

(B) Enlarged zoom of inner spiral bundles as indicated in (A). Scale bar, 3 μm.

(C) Enlarged zoom of outer spiral bundles as indicated in (A). Scale bar, 3 μm.

(D) High-resolution image of an auditory nerve terminal (green) associated with a synaptic ribbon (red arrow). The location is indicated in (B). Scale bar, 1 μm.

(E) High-resolution image of efferent (magenta) and afferent terminals (blue) beneath an outer hair cell. The location is indicated in (C). Scale bar, 1 μm.

(Videos S3, S4, and S5). We collected 3 such large SBEM datasets of the organ of Corti from mouse middle cochlear region (Hua et al., 2021; Wang et al., 2021). They were 325 × 204 × 207 μm^3 (CBA-1), 215 × 215 × 148 μm^3 (CBA-2) and 248 × 211 × 149 μm^3 (C57) in size, and acquired at 11 × 11 × 40 nm^3, 12 × 12 × 50 nm^3 and 12 × 12 × 50 nm^3 pixel size, respectively. The numbers of serial sections and acquisition durations were 5193 and 21 days (5 min / section), 2952 and 9 days (3.6 min / section), as well as 2985 and 12 days (4.8 min / section).

LIMITATIONS

The current sample preparation procedure was optimized for mouse cochlea up to 1.5 mm in diameter. For larger samples for instance cochlea of guinea pig, cat or primate, prolonged durations of

perfusion, staining, dehydration, and embedding are critical for homogeneousmembrane contrast and resin sectioning quality. Few iterations may be required to optimize the experimental conditions.

The maximum allowed sample dimension for commercial SBEM setups is currently limited to 1 mm in xy by the width of the equipped ultramicrotome knife and few hundred microns in z by the motor range of the microtome stage. The automated tape-collecting ultramicrotome (ATUM, (Baena et al., 2019; Hayworth et al., 2014)) offers an alternative approach for imaging larger samples, for which, however, the stack alignment in three dimension becomes more challenging due to mechanical distortions of thin sections.

In comparison with focused ion beam (FIB-) SEM (Knott et al., 2008; Xu et al., 2017), which can achieve isotropic nanoscale resolution up to 5 × 5 × 5 nm^3, SBEM yields slightly worse z-resolution due to mechanical cutting (typically 25–30 nm for resin-embedded biological samples (Briggman and Bock, 2012; Titze and Genoud, 2016) and <14 nm for aluminium (Mancuso et al., 2010)). Hybrid methods including ATUM-FIB (Kislinger et al., 2020), multi-energy deconvolution SBEM (de Goede et al., 2017) have been proposed to meet the needs of simultaneous high resolution and large volume EM imaging.

TROUBLESHOOTING

Problem 1

Poor ultrastructure preservation.

Potential solution 1

1. (Most important) handle the decalcified sample gently during transferring. We use disposable Pasteur pipette for transferring sample between solutions.
2. Adapt the perfusion flow rate (step 2, e) and post-fixation duration (step 2, h) to the sample size. This protocol is optimized for cochlea of adult mouse and considers using greater flow rate and prolonged post-fixation for other species with larger cochleae.

Problem 2

Sample contamination by heavy metal precipitates

Potential solution 2

1. (Most important) use freshly prepared lead solution (step 13) with precisely adjusted pH.
2. In our hands heavy metal precipitates were observed if the sample was not properly perfused (step 2) or washed before the heavy metal staining (step 5). Consider adding more wash steps (step 4).

   Note: In this protocol, we excluded the staining step with uranyl acetate, because in our hands the reagent always leads to precipitation in *en bloc* stained cochlea.

Problem 3

Poor cutting quality.

Potential solution 3

1. (Most important) always use freshly prepared resin (step 18), ideally with newly opened kit.
2. Use new diamond knife before starting a large image stack and increase the cutting thickness or FCC gas flow when the image quality deteriorates due to bad cutting (step 32).

3. Poor cutting could also be a consequence of knife damage by an incompletely decalcified sample. Consider replacing the EDTA solution during the decalcification (step 3).

Problem 4

Uneven staining periphery *vs.* central parts.

Potential solution 4

1. Poor staining homogeneity could be due to insufficient tissue permeability or exhausted stains. Consider replacing the EDTA solution in prolonged decalcification and increasing the volume of staining solutions for large cochlea samples.

Problem 5

Inhomogeneous focus quality.

Potential solution 5

1. Correct EM astigmatism.
2. Increase FCC gas flow or place the FCC carbon needle closer to the block-face.
3. Use small frame size and do in-plane stitching.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yunfeng Hua (yunfeng.hua@shsmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets generated during this study are available at https://wklink.org/9870.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100515.

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AUTHOR CONTRIBUTIONS

Y.H. supervised the study. Y.L., F.W., H.W., and P.B. conducted the experiment. Y.H. and Y.L. drafted the manuscript with the help of all other authors.

DECLARATION OF INTERESTS

P.B. is employed by Zeiss Research Microscopy Solutions. All other authors have no conflicts of interest to declare.

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