

Mouse Cochlear Whole Mount Immunofluorescence

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[Abstract] This protocol comprises the entire process of immunofluorescence staining on mouse cochlea whole mount, starting from tissue preparation to the mounting of the tissue. This technique provides “three-dimensional” views of the stained components in order to determine the localization of a protein of interest in the tissue in its natural state and environment.

Materials and Reagents

1. Ketamine hydrochloride (100 mg/ml) (Ketaset FORT DODGE)
2. Xylazine hydrochloride (100 mg/ml) (Xylazine AnaSed)
3. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: P6148)
4. Sodium Phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: S9638)
5. Sodium Phosphate heptahydrate ($\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: S9390)
6. Sodium Hydroxide (NaOH) (Thermo Fisher Scientific, catalog number: BP359)
7. Sodium Chloride (NaCl) (Sigma-Aldrich, catalog number: S3014)
8. Phosphate buffer (PB)
9. Phosphate buffer saline (PBS)
10. EDTA (Sigma-Aldrich, catalog number: E7889)
11. Blocking buffer
12. Normal goat serum (NGS) (Sigma-Aldrich, catalog number: G-6767)
13. Saponin (Sigma-Aldrich, catalog number: 47036)
14. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
15. Primary Antibodies
 - a. Rabbit anti-myosin VIIa antibody (a hair cell-specific marker) (Proteus Biosciences, catalog number: 25-6790) at a dilution of 1:50 in blocking buffer.
 - b. Guinea pig anti- VGLUT3 (Vesicle Glutamate Transporter 3) antibody at 1:5,000 in blocking buffer (a gift from Dr. Robert Edward, Department of Neurology, UCSF)
16. Secondary Antibodies

- a. Cy2-conjugated Goat anti-rabbit secondary IgG antibody (Jackson ImmunoResearch) at a dilution of 1:4,000 in PBS.
 - b. Cy3-conjugated Goat anti-guinea pig secondary IgG antibody (Jackson ImmunoResearch) at a dilution of 1:4,000 in PBS.
- Note: Secondary antibodies should be diluted in PBS (not blocking buffer)*
17. DAPI to counterstain nuclei (1.5 µg/ml in PBS) (Sigma-Aldrich, catalog number: D9564).
 18. Antifade FluorSave reagent (Calbiochem, catalog number: 34589)
 19. 10x PB (see Recipes)
 20. 10x PBS (see Recipes)
 21. 4% PFA-PB (50 ml) (see Recipes)
 22. Blocking buffer (see Recipes)

Equipment

1. Syringe (1 ml syringe 25G1(BD Biosciences, catalog number: 329622)
2. 0.5 ml microcentrifuge tube's caps ((Thermo Fisher Scientific, catalog number: 02-681-311)
3. Microdissecting scissor (Fine Science Tools, catalog number: 15002-08),
4. Fine forceps (Ted Pella, catalog number: 5621)
5. Surgical blade (Feather)
6. 5 ml Vials (Denville, catalog number: T8200-S).
7. Pasteur pipettes (used to rinse the tissue) (Thermo Fisher Scientific, catalog number: 13-678-20)
8. SuperFrost slides (Thermo Fisher Scientific, catalog number: 12-550)
9. Cover slips (Thermo Fisher Scientific, catalog number: 12-548-5A)
10. Petri dish (BD Biosciences, Falcon[®], catalog number: 1007)
11. Aluminum foil
12. Refrigerator
13. Rocking platform
14. Dissecting microscope
15. Confocal microscope

Procedure

- A. Cochlea extraction and perfusion
 1. Anesthetize mice (mice overdosed with 200 µl of a mixture of ketamine hydrochloride, 100 mg/ml and xylazine hydrochloride 10 mg/ml, per 100 g of mouse weight).

2. Pinch the toes to check if the animal is fully asleep then decapitate the mouse.
3. Quickly and carefully remove cochleae from skull and put them in a Petri dish as follow:
 - a. Cut the mouse head in half.
 - b. Remove brain.
 - c. Hold head on side so the 'bowl' of the skull is facing up.
 - d. The temporal bone is in the back and is not continuous to the skull (there are many small bones attaching it to the skull).
 - e. Push in the junctions between the skull and the temporal bone (away from the cochlea area) to break it away from skull.
 - f. Carefully dissect extraneous bones away from the cochlea.

The following video by Parker *et al.* (2010) shows, between min 3 and 4, how to dissect the temporal bone (cochlea) from a P3-5 mouse. The same procedure is done for an older mouse, but the only difference is the bone cochlea becomes harder.

<http://www.jove.com/video/1685/primary-culture-plasmid-electroporation-murine-organ>.

4. Use a syringe to push approximately 0.5 ml of ice cold 4% PFA-PB through the round and oval windows (RW and OW) after having made a small hole in the apex of the cochlea (for orientation refer to the whole cochlea picture with legends in Figure 1 (Glen *et al.*, 2008)).

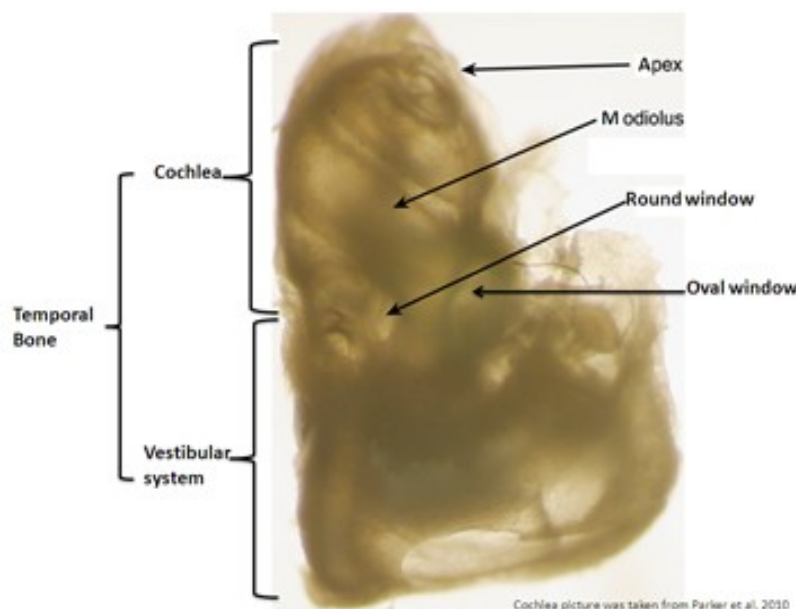


Figure 1. Shows a mouse decalcified left temporal bone. This labeled picture helps for orientation on the mouse anatomy of the whole temporal bone needed for good success in reproducing this protocol [this picture was borrowed from MacDonald *et al.* (2008) paper].

Note: To have a very good fixation, the round window membrane should be punctured and the stapes should be removed before cochlea perfusion.

5. Post-fix the cochleae in 5 ml 4% PFA-PB for 2 h at 4 °C under gentle agitation.
6. Using 1ml syringe rinse cochlea by perfusing 3 times PB through the RW and OW.
7. Wash 3 x 15 min in fresh PB at 4 °C under gentle agitation.

B. Cochlea decalcification and organ of Corti dissection into surface preparation

1. Transfer the cochleae to 5 ml tube containing 5% EDTA and incubate at 4 °C under gentle agitation.

Post-natal Age	< P7	P7-P15	P15-P21	> P21
Time in 5% EDTA	1-2 h	2-4 h	5-8 h	12- 24 h

Note: Before going to next step, you need to check if the cochleae are very well decalcified. Insufficient decalcification will not help to get an intact and good organ of Corti. A good decalcification is when the cochleae become soft at touch with forceps and when the cochlea bone becomes transparent. A picture of decalcified cochlea is present in the following video.

<http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/video-tutorial-for-cochlear-dissection/>

2. Wash 3 x 15 min in fresh PB at 4 °C under gentle agitation.

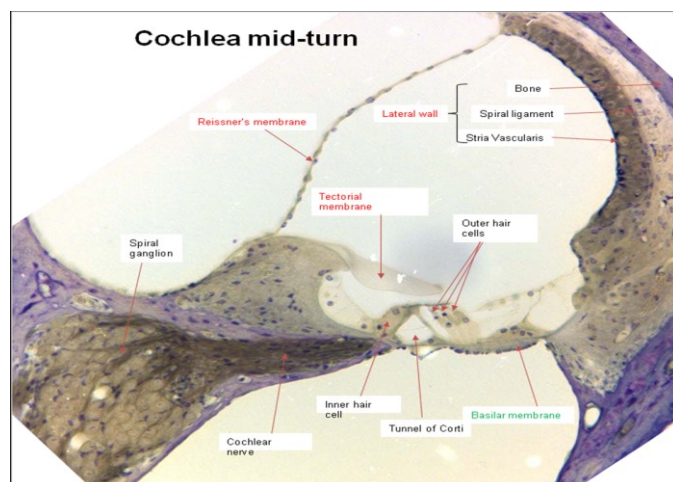


Figure 2. Shows a mid-modular cross section of the mouse cochlea turn stained with toluidine blue. This labeled 20x view section helps for orientation on the mouse cochlea anatomy needed in this protocol for good success in reproducing this protocol.

3. The otic capsule, the lateral wall, Reissner's membrane and tectorial membrane should be removed in that order and then dissect the organ of Corti (for orientation on the cochlea anatomy see the cochlea mid-turn cross section Figure 2).
4. The remaining organ of Corti was further dissected into a surface preparation and microdissected into individual turns.

Note: The video of Liberman's laboratory shows a method of surface preparation dissection that better preserves the overall structure of the organ of Corti.

<http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/video-tutorial-for-cochlear-dissection/>

C. Immunofluorescence

1. Microdissected cochlea individual turns were then placed in a 0.5 ml Ependorf tube cap containing blocking buffer and incubated for 1 h at room temperature (RT) on a rocking platform.
2. Incubate in primary antibodies diluted in blocking buffer overnight at 4 °C on a rocking platform.

Note: Putting the cap with cochlea turns in a small Petri dish and wrapping them with parafilm will prevent dehydration.

3. Rinse 3 x 15 min in PBS.
4. Incubate with secondary antibodies in PBS for 2 h at RT on a rocking platform.
5. Rinse 3 x 15 min in PBS.
6. Stain the nuclei with DAPI in PBS for 15 min at RT.
7. Rinse 3 x 15 min in PBS.
8. Mount the cochlea whole mount turn carefully onto slides.

Note: The orientation is very important in mounting the whole mount organ of Corti. The hair cells stereocilia should face up and be in contact with the coverslip, while the basilar membrane labeled green in Figure 2 should be on the bottom and be in contact with the slide. The video of Liberman's laboratory shows the correct orientation of the cochlea surface preparation a slide of the video is presented in Figure 3.

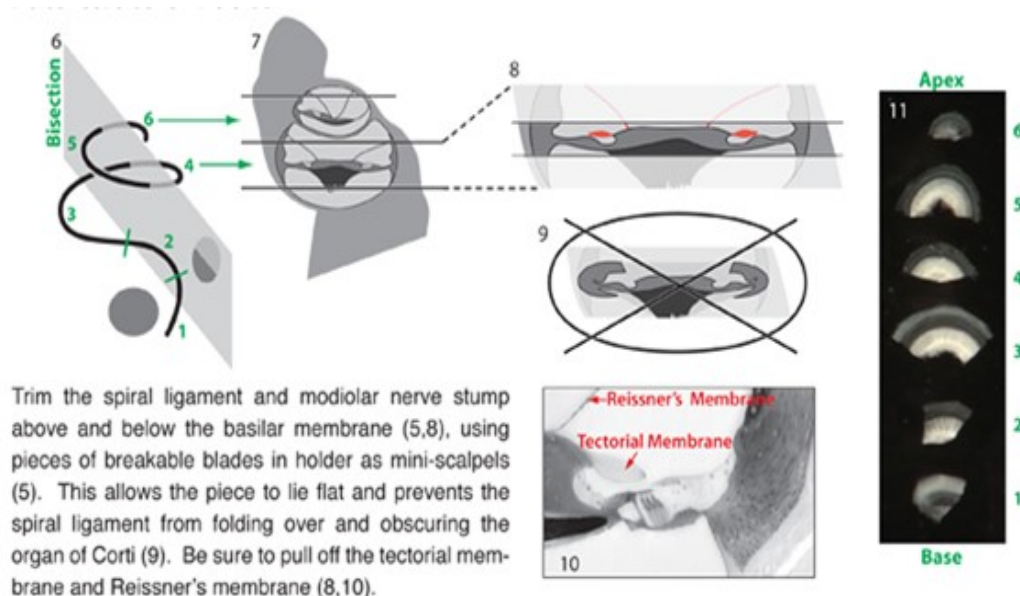


Figure 3. Shows a slide of the video of Liberman's laboratory.

["http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/video-tutorial-for-cochlear-dissection/"](http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/video-tutorial-for-cochlear-dissection/) presenting the correct orientation and preparation of the whole mount organ of Corti. The hair cells stereocilia should face up and be in contact with the coverslip, while the basilar membrane labeled green in Figure 2 should be on the bottom and be in contact with the slide. At this step if the orientation of the cochlea surface preparation is done properly it provides very good views of the stained components under the microscope.

9. Apply a drop of antifades solution, coverslip and then let it dry.
10. Slides are now ready to be observed under a microscope equipped with epifluorescence.

Note: For better images use a confocal immunofluorescence microscope and do not expose your slides too much to light to avoid stain bleaching.
11. Figure 2 in Akil *et al.* (2012) Neuron paper is a sample of the Mouse Cochlear Whole Mount Immunofluorescence.

Recipes

1. 10x PB

Dissolve 0.23 g anhydrous NaH_2PO_4 and 2.17 g Na_2HPO_4 heptahydrate into 80 ml ddH₂O. Adjust pH to 7.4 and bring the final volume to 100 ml.
2. 10x PBS

Dissolve 0.23 g anhydrous NaH_2PO_4 and 2.17 g Na_2HPO_4 heptahydrate, and 8.75 g NaCl into 80 ml ddH₂O. Adjust pH to 7.4 and bring the final volume to 100 ml.

3. 4% PFA-PB (50 ml)

Dissolve 2 g PFA in approximately 40 ml in ddH₂O.

(Note: 1-You will need to heat the water and add NaOH to dissolve the PFA. 2- Keep the temperature below 60 °C during the entire process to avoid breakdown of the PFA polymers).

After the PFA is dissolved, add 5 ml of 10x PB. Cool to RT and adjust the pH to 7.4. Bring the final volume to 100 ml. Filter and store at 4 °C.

Note: Use this solution within a week or less.

4. Blocking buffer

20% normal goat serum (NGS) with 0.3% TritonX-100 and 0.3% Saponin in PBS

Acknowledgments

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References

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