

Submission ID #: 72562

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Title: Whole-Mount Dissection of the Vestibular Complex and Quantification of Vestibular Ganglion Neurons in Mice

Authors and Affiliations:

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera? **No**

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces, and **you will have to perform the procedure using one eye.**

Leica M80: Teaching stereo dissection microscope with 2 sets of objectives

SCOPE Shots: 3.3.2, 3.3.3, 4.1.3, 4.2.1, 4.2.2, 4.3.1, 4.3.2, 4.4.1, 4.4.2, 4.4.3, 4.4.4, 4.5.1, 4.5.2, 4.6.1, 4.6.2, 5.1.4, 5.1.5, 5.2.1, 5.2.2, 5.3.1.

Videographer : Please film the above-mentioned shots using a SCOPE kit.

*Videographer: Please note, that the authors would like images of the temporal bone in 2 orientations in **SCOPE** shot **4.2.1**.*

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **Yes, Same building, rooms are a floor apart**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 17

Number of Shots: 37

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Debbie O'Bryan:** We examine vestibular organization and connectivity, asking how neuron numbers, projections, and ganglion size change with development, damage, and aging.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Debbie O'Bryan:** Previous whole-mount methods preserve only sensory epithelia, limiting neuronal analysis. Our protocol retains the vestibular ganglion and all five connected organs.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Brandon Cox:** Our method enables researchers to visualize dendritic projections, measure vestibular ganglion size, and quantify neuronal cell bodies in three dimensions.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Debbie O'Bryan:** The main challenge is preventing the complex from twisting or folding during mounting because its interconnected structures have different orientations.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Brandon Cox:** Future studies can investigate neuronal differentiation during development and changes in vestibular neurons, projections, and innervation patterns following aging or injury.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Southern Illinois University School of Medicine

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

NOTE: Audio slate was used for most shots since they were close up and there wasn't room for hard slate. The author prefers the last take on shots with multiple takes.

Scope kit was not compatible with the lab scope. As a result, many shots had to be handheld. For future shoots, having Jove provide a compatible scope kit with camera or confirming compatibility would be helpful. We did a 2nd take of several scope shots at the end of the shoot for the editor.

2. Extraction and Fixation of Temporal Bones

Demonstrator: Deborah O'Bryan

2.1. After removing the brain from the mouse's skull, locate the temporal bones at the base of the skull [1]. Place one thumb at the tip of the otic capsule and press down on the posterior semicircular canal with the other thumb to separate the encapsulated temporal bone from the skull [2]. Free the lower half of the temporal bone with the thumb and index finger [3].

2.1.1. WIDE: Talent locating the temporal bones at the base of the skull. NOTE: 2.1.1. and 2.1.2 is filmed in a single shot

2.1.2. Talent separating the encapsulated temporal bone by pressing on the posterior semicircular canal.

2.1.3. CU: Talent freeing the lower half of the temporal bone manually.

Added shot: Close-up of the whole temporal bones

2.2. Place both temporal bones from one mouse in a 2-milliliter microcentrifuge tube containing 1 milliliter of 4% paraformaldehyde prepared in 10 millimolar PBS [1]. Incubate at room temperature for 20 to 24 hours [2].

2.2.1. CU: Talent transferring both temporal bones into individual 2-milliliter tubes containing 1 milliliter of paraformaldehyde.

2.2.2. Shot of the labeled tube containing the temporal bones being left at room temperature for 20 to 24 hours.

3. Decalcification of Temporal Bones

- 3.1. After fixing the temporal bones, use a pipette to remove the paraformaldehyde [1] and replace it with 120 millimolar EDTA, filling the 2-milliliter microcentrifuge tube completely, with slightly more than 2 milliliters of EDTA, to prevent air bubbles when the tube is closed [2].
 - 3.1.1. CU: Talent discarding PFA from the tube with a pipette.
 - 3.1.2. ECU: Talent filling the 2-milliliter microcentrifuge tube completely with EDTA solution to avoid air bubbles.
- 3.2. Place the tube on an end-over-end rotator at 4 rpm and room temperature for at least 3 days [1]. Replace the EDTA with a fresh solution approximately every 24 hours [2].
 - 3.2.1. WIDE: Talent placing the tube on an end-over-end rotator set to 4 rpm at room temperature.
 - 3.2.2. Shot of old EDTA solution being replaced with fresh solution.
- 3.3. To evaluate decalcification, place a temporal bone in a silicone elastomer-coated dissection dish [1] and gently press the vestibule with forceps [2]. Verify whether the bone has a spongy texture, indicating the end of the decalcification process [3]. Once decalcified, replace the EDTA with 500 to 1,000 microliters of 10 millimolar PBS [4], and store at 4 degrees Celsius until dissection [5].
 - 3.3.1. Talent placing the temporal bone in a silicone elastomer-coated dish
 - 3.3.2. SCOPE: Talent gently pressing the vestibule with forceps.
 - 3.3.3. SCOPE: The adequately decalcified, spongy temporal bone yielding gently under the forceps.
 - 3.3.4. Talent replacing the EDTA solution with 10 millimolar PBS. **NOTE: Not filmed, Reuse 3.2.2**
 - 3.3.5. Talent storing the sample at 4 degrees Celsius until dissection.

4. Whole-Mount Dissection of the Vestibular Complex

- 4.1. Fill a silicone elastomer-coated dissection dish to two-thirds with 10 millimolar PBS [1]. Place one decalcified temporal bone in the dish under a stereo-dissection microscope for dissection [2]. Use number 4 or 5 straight jeweler's forceps and 5-millimeter ultrafine microsurgical spring scissors to remove excess muscle tissue [3].
 - 4.1.1. WIDE: Talent filling the silicone elastomer-coated dish with PBS.

- 4.1.2. Talent positioning the dish with one decalcified temporal bone under the stereo-dissection microscope.
- 4.1.3. SCOPE: Talent removing excess muscle tissue with jeweler's forceps and 5-millimeter ultrafine microsurgical spring scissors.
- 4.2. Hold the outer edges of the temporal bone with number 4 or 5 straight jeweler's forceps, with the cochlea facing down [1]. With a second pair of forceps, grasp and gently peel away the thin piece of bone running perpendicular to the vestibule [2].
 - 4.2.1. SCOPE: Talent holding the temporal bone with the cochlea facing down.
 - 4.2.2. SCOPE: Talent gently peeling away the thin bone perpendicular to the vestibule.
- 4.3. Using 5-millimeter ultrafine microsurgical spring scissors, remove the superior portion of the anterior semicircular canal, including any excess brain tissue [1]. Cut away the apical and middle portions of the cochlea, leaving at least half of the basal turn, approximately 1.5 millimeters, to protect the inferior vestibular ganglion [2].
 - 4.3.1. SCOPE: Talent removing the superior anterior semicircular canal area and excess brain tissue.
 - 4.3.2. SCOPE: Talent cutting away the apical and middle cochlea while retaining at least half of the basal turn.
- 4.4. Place one scissor blade inside the temporal bone and cut along the vestibular endolymphatic duct, continuing through the cochlea [1]. Then, place one blade inside the duct and make a second cut along the anterior side of the vestibule [2]. Carefully cut away the membranous labyrinth covering the utricle [3]. Use a curved 2.5-millimeter Vannas spring scissors to trim the remaining temporal bone covering the tops of the three cristae [1].
 - 4.4.1. SCOPE: Talent cutting along the vestibular endolymphatic duct all the way through to the cochlea.
 - 4.4.2. SCOPE: Talent making the second cut from the duct along the anterior side of the vestibule.
 - 4.4.3. SCOPE: Talent cutting away the membranous labyrinth over the utricle without disturbing the sensory epithelium.
 - 4.4.4. SCOPE: Talent trimming the bone over the three cristae with curved 2.5-millimeter Vannas spring scissors.

- 4.5. Using a blunt 26-gauge needle attached to a 1-milliliter insulin syringe as a handle, slowly coax each crista out of its bone casing [1]. Then, use 5-millimeter ultrafine microsurgical spring scissors to cut away the bone that held the cristae [2].

4.5.1. SCOPE: Talent slowly coaxing each crista out of the bone casing with the blunt 26-gauge needle. NOTE: 4.5.1. and 4.5.2 are filmed in a single shot

4.5.2. SCOPE: Talent cutting away the exposed bone around the released cristae.

- 4.6. Use curved 2.5-millimeter Vannas spring scissors to trim the remaining basal cochlea and any residual tissue or bone around the vestibular complex [1]. Handle the completed whole mount only by a membranous labyrinth attached to a crista and avoid pulling on the ganglion or sensory epithelia [2].

4.6.1. SCOPE: Talent trimming the remaining basal cochlea and residual tissue from the intact vestibular complex. NOTE: 5.6.1 and 5.6.2 are filmed in a single shot

4.6.2. SCOPE: Talent gently handling the completed whole mount by the membranous labyrinth at the crista. NOTE: Filled 2nd take with vestibular complex filled over. Called it 4.6.2 reverse

5. Mounting the Vestibular Complex on Slides

- 5.1. Label each microscope slide with the sample identification and antibodies used [1]. Place an imaging spacer on the slide [2-TXT]. Slowly, pipette approximately 20 microliters of mounting medium into the center of the spacer, avoiding air bubbles [3]. Under the stereo-dissection microscope, use number 4 or 5 straight jeweler's forceps to lift the tissue by a membranous labyrinth attached to one of the cristae [4]. Place the tissue in the center of the spacer [5].

5.1.1. CU: Talent labeling a microscope slide with the sample ID and antibody used.

5.1.2. Talent positioning the imaging spacer on the slide. **TXT – Imaging Spacer dimensions: Diameter = 13 mm ; Depth = 0.12 mm**

5.1.3. CU: Talent slowly dispensing mounting medium into the spacer without introducing bubbles.

5.1.4. SCOPE: Talent lifting the immune-stained tissue by a membranous labyrinth connected to a crista. NOTE: 5.1.4 and 5.1.5 are filmed in a single shot

5.1.5. SCOPE: Talent placing the vestibular complex in the center of the spacer.

- 5.2. For imaging of the vestibular ganglion, orient the utricular sensory epithelium upwards [1]. For imaging neuronal projections, orient the utricular sensory epithelium facing down [2].

- 5.2.1. SCOPE: The vestibular complex oriented with the utricular sensory epithelium facing up. **TXT - Ganglion view** **NOTE: Filmed with 4.6.2**
- 5.2.2. SCOPE: The vestibular complex oriented with the utricular sensory epithelium facing down. **TXT - Neuronal projection view** **NOTE: Filmed with 4.6.2 reverse**
- 5.3. Gently apply a number 1.5 coverslip by pointing the three cristae away from the lowering edge, placing one side on the slide, and slowly angling it down over the tissue to minimize folding [1].
 - 5.3.1. SCOPE: Talent lowering one edge of a number 1.5 coverslip gradually over the oriented tissue without folding the vestibular organs.

Results

6. Results

6.1. The dissection and imaging method enabled counting of β -III (*beta three*) tubulin-positive superior vestibular ganglion neurons at 40X (*forty ex*) magnification [1]. It also enabled visualization and tracing of neuronal projections extending to the posterior crista [2].

6.1.1. LAB MEDIA: Figure 5 **Video Editor:** *In Panel A on the left, please highlight the white dashed box and the red X marking a green neuron.*

6.1.2. LAB MEDIA: Figure 5 **Video Editor:** *In Panel B on the right, please highlight the green fiber-like neuronal projections.*

6.2. A common dissection error occurred at the natural weak point of the dumbbell-shaped vestibular ganglion, causing the superior and inferior regions to separate [1]. Representative confocal projections showed that part of the inferior vestibular ganglion [2] and the posterior crista had been torn away [3].

6.2.1. LAB MEDIA: Figure 6 **Video Editor:** *Please highlight the dashed circle at the lower-right of the merged image in Panel A, with a glowing outline.*

6.2.2. LAB MEDIA: Figure 6 **Video Editor:** *Please use an arrow to point to the disrupted green tissue inside the dashed circle, in Panel B.*

6.2.3. LAB MEDIA: Figure 6 **Video Editor:** *Please highlight with an arrow the missing or damaged red tissue inside the dashed circle, in Panel C.*

6.3. A mounting error occurred when the saccule folded over the vestibular ganglion, obscuring the underlying neuronal cell bodies, as observed in the merged [1], TuJ1 (*tee-u-jay-one*) [2], and myosin VIIa (*seven-a*) channels [3].

6.3.1. LAB MEDIA: Figure 7 **Video Editor:** *In Panel A, please highlight the white dashed circle showing the overlapping tissue.*

6.3.2. LAB MEDIA: Figure 7 **Video Editor:** *In Panel B, use an arrow to highlight the green neuronal region obscured inside the circle.*

6.3.3. LAB MEDIA: Figure 7 **Video Editor:** *In Panel C, use an arrow to highlight the red folded tissue inside the circle.*

7. Temporal
Pronunciation link: <https://www.merriam-webster.com/dictionary/temporal>
IPA: /'tempərəl/
Phonetic Spelling: TEM·puh·ruhl
8. Otic
Pronunciation link: <https://www.merriam-webster.com/dictionary/otic>
IPA: /'oʊtɪk/
Phonetic Spelling: OH·tik
9. Posterior
Pronunciation link: <https://www.merriam-webster.com/dictionary/posterior>
IPA: /pɑː'stɪriər/
Phonetic Spelling: pah·STEER·ee·er
10. Semicircular
Pronunciation link: <https://www.merriam-webster.com/dictionary/semicircular>
IPA: /,semi'sɜːkjələr/
Phonetic Spelling: sem·ee·SUR·kyuh·ler
11. Microcentrifuge
Pronunciation link: No confirmed link found
IPA: /,maɪkroʊ'sentrə,fjuːdʒ/
Phonetic Spelling: my·kroh·SEN·truh·fyooj
12. Paraformaldehyde
Pronunciation link: No confirmed link found
IPA: /,pærəfɔːr'mældə,haɪd/
Phonetic Spelling: pair·uh·for·MAL·duh·hyde
13. Millimolar
Pronunciation link: No confirmed link found
IPA: /,mɪlɪ'moʊlər/
Phonetic Spelling: mil·ih·MOH·ler
14. Decalcification
Pronunciation link: <https://www.merriam-webster.com/dictionary/decalcification>
IPA: /diː,kælsəfə'keɪʃən/
Phonetic Spelling: dee·kal·suh·fuh·KAY·shuhn
15. EDTA
Pronunciation link: <https://www.merriam-webster.com/dictionary/EDTA>
IPA: /,iː diː tiː 'eɪ/
Phonetic Spelling: E·D·T·A
16. Elastomer
Pronunciation link: <https://www.merriam-webster.com/dictionary/elastomer>
IPA: /iː'læstəmər/
Phonetic Spelling: ee·LAS·tuh·mer
17. Vestibule
Pronunciation link: <https://www.merriam-webster.com/dictionary/vestibule>
IPA: /'vestə,bjuːl/
Phonetic Spelling: VES·tuh·byool

- 18. Vestibular**
Pronunciation link: <https://www.merriam-webster.com/dictionary/vestibular>
IPA: /ve'stɪbjələr/
Phonetic Spelling: veh·STIB·yuh·ler
- 19. Microsurgical**
Pronunciation link: No confirmed link found
IPA: /ˌmaɪkroʊ'sɜːdʒɪkəl/
Phonetic Spelling: my·kroh·SUR·jih·kuhl
- 20. Cochlea**
Pronunciation link: <https://www.merriam-webster.com/dictionary/cochlea>
IPA: /'kɑːkliə/
Phonetic Spelling: KOK·lee·uh
- 21. Perpendicular**
Pronunciation link: <https://www.merriam-webster.com/dictionary/perpendicular>
IPA: /ˌpɜːpən'dɪkjələr/
Phonetic Spelling: per·puhn·DIK·yuh·ler
- 22. Apical**
Pronunciation link: <https://www.merriam-webster.com/dictionary/apical>
IPA: /'eɪpɪkəl/
Phonetic Spelling: AY·pih·kuhl
- 23. Basal**
Pronunciation link: <https://www.merriam-webster.com/dictionary/basal>
IPA: /'beɪsəl/
Phonetic Spelling: BAY·suhl
- 24. Ganglion**
Pronunciation link: <https://www.merriam-webster.com/dictionary/ganglion>
IPA: /'gæŋɡliən/
Phonetic Spelling: GANG·glee·uhn
- 25. Endolymphatic**
Pronunciation link: <https://www.merriam-webster.com/medical/endolymphatic>
IPA: /ˌendəʊlɪm'fætɪk/
Phonetic Spelling: en·doh·lim·FAT·ik
- 26. Membranous**
Pronunciation link: <https://www.merriam-webster.com/dictionary/membranous>
IPA: /'membrənəs/
Phonetic Spelling: MEM·bruh·nuhs
- 27. Labyrinth**
Pronunciation link: <https://www.merriam-webster.com/dictionary/labyrinth>
IPA: /'læbəˌrɪnθ/
Phonetic Spelling: LAB·uh·rinth
- 28. Utricle**
Pronunciation link: <https://www.merriam-webster.com/dictionary/utricle>
IPA: /'juːtrɪkəl/
Phonetic Spelling: YOO·trih·kuhl

- 29. Epithelium**
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelium>
IPA: /ˌepəˈθiːliəm/
Phonetic Spelling: ep·uh·THEE·lee·uhm
- 30. Cristae**
Pronunciation link: <https://www.merriam-webster.com/medical/crista>
IPA: /ˈkrɪstiː/
Phonetic Spelling: KRIS·tee
- 31. Crista**
Pronunciation link: <https://www.merriam-webster.com/medical/crista>
IPA: /ˈkrɪstə/
Phonetic Spelling: KRIS·tuh
- 32. Vannas**
Pronunciation link: No confirmed link found
IPA: /ˈvænəs/
Phonetic Spelling: VAN·uhs
- 33. Utricular**
Pronunciation link: <https://www.merriam-webster.com/medical/utricular>
IPA: /juːˈtrɪkjələr/
Phonetic Spelling: yoo·TRIK·yuh·ler
- 34. Neuronal**
Pronunciation link: <https://www.merriam-webster.com/dictionary/neuronal>
IPA: /nʊˈroʊnəl/
Phonetic Spelling: noo·ROH·nuhl
- 35. Coverslip**
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>
IPA: /ˈkʌvərˌslɪp/
Phonetic Spelling: KUV·er·slip
- 36. Tubulin**
Pronunciation link: <https://www.merriam-webster.com/dictionary/tubulin>
IPA: /ˈtuːbjəlɪn/
Phonetic Spelling: TOO·byuh·lin
- 37. Confocal**
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kɑːnˈfoʊkəl/
Phonetic Spelling: kon·FOH·kuhl
- 38. Saccule**
Pronunciation link: <https://www.merriam-webster.com/dictionary/saccule>
IPA: /ˈsækjuːl/
Phonetic Spelling: SAK·yool
- 39. Myosin**
Pronunciation link: <https://www.merriam-webster.com/dictionary/myosin>
IPA: /ˈmaɪəsin/
Phonetic Spelling: MY·uh·sin

