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Title: In Vitro Wedge Slice Preparation for Mimicking In Vivo Neuronal Circuit Connectivity

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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 similar? **Y**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **51**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Catherine Weisz**: Although brain slice experiments allow neuronal function interrogation with a high electrical and temporal resolution, these slices typically sever many presynaptic connections. A wedge-shaped slice maintains a more intact presynaptic circuitry **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Matthew Fischl**: This modified brain slice maintains a more complete, in vivo-like neuronal circuit, while providing the benefits of in vitro experimentation, such as visually guided patch-clamp recordings, pharmacology, and activity imaging **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Matthew Fischl:** Accurate wedge slice geometry can be estimated based on the atlas locations of the neurons within the circuit, but the integrity of the presynaptic somata and axons should be determined using histology **[1]**

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the National Institute on Deafness and Other Communication Disorders.

Protocol

2. Brain Removal with Intact Auditory Nerve Root

2.1. Begin by using a razor blade to cut the skin at the midline of the skull from the nose to the back of the neck **[1-TXT]**.

2.1.1. WIDE: Talent cutting skin **TEXT: Euthanasia: CO_2 asphyxiation and decapitation**

2.2. Peel back the skin to expose the skull **[1-TXT]** and, starting at the base of the skull and continuing towards the nose, use small scissors to make an incision in the skull through the midline **[2]**.

2.2.1. Skin being peeled **TEXT: See text for transgenic mouse generation details**

2.2.2. Incision being made

2.3. At the lambda suture, make cuts in the skull from the midline laterally toward the ear on both sides to peel back the skull to expose the brain **[1]**.

2.3.1. Cuts being made/skull being peeled
2.3.2. Skull being peeled

2.4. Starting at the rostral end, use a small lab spatula to gently lift the brain from the skull to allow the optic nerve to be severed **[1]**.

2.4.1. Brain being lifted/nerve being cut
2.4.2. Nerve being cut

2.5. Continue to gently work the brain backwards, exposing the ventral surface **[1]**, and use fine forceps to carefully pinch the trigeminal nerves near the ventral surface of the brainstem to cut them **[2]**.

2.5.1. Ventral surface being exposed
2.5.2. Nerves being pinched

2.6. Place the preparation in a glass Petri dish filled with cold slicing solution **[1-TXT]** and place the dish under a dissecting microscope **[2-TXT]**.

2.6.1. Talent placing preparation into dish **TEXT: See text for all solution preparation details**
2.6.2. Dish under microscope being bubbled **TEXT: Gently bubble with carbogen**

2.7. Trim the facial nerve close to the brainstem to expose the vestibulocochlear nerve **[1]**.

2.7.1. SCOPE: Nerve being trimmed
2.7.2. SCOPE: Tips being pushed into foramina NOTE: Shot moved to next step

2.8. Use fine forceps to push the tips into the foramina where the vestibulocochlear nerve exits the skull as far as possible **[2.7.2]**. Pinch to sever the nerves**[1]** on both sides, leaving the nerve root attached to the brainstem **[2]**

2.8.1. SCOPE: Nerve being pinched
2.8.2. SCOPE: Meninges being removed NOTE: Shot moved to next step

2.9. Remove the meninges and vasculature from the ventral surface of the brainstem near the trapezoid body **[2.8.1]**. Then pinch the remaining cranial nerves and connective tissue to free the brain completely from the skull, taking care to preserve the remaining spinal cord if possible **[1]**.

2.9.1. SCOPE: Nerves being pinched/brain being freed

3. Brain Tissue Blocking and Mounting

3.1. To prepare the surface of the brain for fixture to the stage, place the brain ventral side up **[1]** and use a blunt tool to gently immobilize the spinal cord to stabilize the brain tissue **[2]**.

3.1.1. WIDE: Talent placing brain ventral side up
3.1.2. SCOPE: Spine being immobilized

3.2. Insert open forceps at an approximately 20-degree angle through the brain to the bottom of the dish so that the tips exit the dorsal surface of the brain caudal to the optic chiasm **[1]** and use a razor blade to cut along the forceps **[2]**.

3.2.1. SCOPE: Forceps being inserted
3.2.2. SCOPE: Tissue being cut

3.3. Next, prepare a 1-cubic centimeter block of 4% agar **[1]** and spread a small drop of glue into a rectangle on the stage **[2]**.

3.3.1. Talent cutting block of agar

3.3.2. Glue being spread

3.4. Use forceps to carefully lift the brain **[1]** and gently dab the excess liquid with the edge of a paper towel **[2]**.

3.4.1. Brain being lifted
3.4.2. Liquid being dabbed

3.5. Then place the blocked surface onto the glue so that the ventral surface will be facing the direction of the blade during slicing **[1]** and push agar block gently against the dorsal surface as a support **[2]**.

3.5.1. Brain being placed onto glue
3.5.2. Agar being pushed against brain

4. Wedge Slice Generation

4.1. To acquire wedge slices with the cochlear nerve root on the thick side and the medial olivocochlear neurons and the medial nucleus of the trapezoid body on the thin side, place the magnetic disc with the attached brain onto the stage holder **[1]** and place the holder in the slicing chamber of a vibratome with the ventral surface of the brain oriented toward the blade **[2]**.

4.1.1. WIDE: Talent placing disc onto stage holder
4.1.2. Talent placing holder into chamber

4.2. Fill the chamber with ice cold slicing solution **[1]** and lower the blade into the carbogen-bubbled solution **[2]**.

4.2.1. Talent filling chamber with solution
4.2.2. Blade being lowered

4.3. Cut slices caudal to the region of interest to make sure the slices are symmetrical **[1-TXT]**, then shift the stage approximately 15 degrees to one side **[2]**.

4.3.1. Slice being cut **TEXT: If slice asymmetrical, slightly tilt stage to obtain symmetry**
4.3.2. Talent shifting stage

4.4. Continue slicing carefully until the auditory nerve root is close to the surface on one side **[1]** and the facial nerve can be seen at the surface of the other side of the slice **[2]**.

4.4.1. Shot of slice *Videographer: Important/difficult step; Video Editor: please emphasize auditory nerve root*
4.4.2. Use 4.4.1. Shot of slice *Video Editor: please emphasize facial nerve*

4.5. Shift the stage 15 degrees back to the original position **[1]** and move the blade away from the tissue **[2]**.

4.5.1. Stage being shifted *Videographer: Important step*
4.5.2. Blade being moved *Videographer: Important step*

4.6. Spin the stage base 90 degrees so that the lateral edge of the thin side is facing the blade **[1]** and lower the blade several hundred microns **[2]** before slowly bringing the blade close to the edge of the tissue **[3-TXT]**.

4.6.1. Stage base being spun *Videographer: Important step*
4.6.2. Blade being lowered *Videographer: Important step*
4.6.3. Blade being brought close to tissue *Videographer: Important step* **TEXT: Repeat until blade touches later edge of tissue**

4.7. With the blade retracted slightly, lower the blade to the desired thickness of the thin edge of the slice **[1]**.

4.7.1. Blade being retracted/lowered *Videographer: Important/difficult step*

4.8. Move the blade back from the tissue **[1]** and spin the stage base back so that the ventral surface faces the stage base **[2]**.

4.8.1. Blade being moved back
4.8.2. Stage being spun

4.9. Make a cut to designate the rostral surface of the wedge slice **[1]** and transfer the slice to a 1-square centimeter piece of interface paper caudal surface down **[2]**.

4.9.1. Cut being made
4.9.2. Slice being placed onto paper

4.10. The facial nerve should be visible on both hemispheres of the slice on the rostral surface **[1]**.

4.10.1. SCOPE: Shot of slice with visible facial nerve

4.11. Then move the slice to a 35-degree Celsius incubation chamber to recover for 30 minutes **[1]**.

4.11.1. Talent placing slice into incubator

5. Electrophysiology Setup and Recording

5.1. To set up the wedge slice for electrophysiology analysis, place the sample into a recording chamber that is being continuously perfused with 35-degree Celsius aCSF (A-C-S-F) **[1]** and stabilize the slice **[2]**.

5.1.1. WIDE: Talent placing wedge into recording chamber
5.1.2. Slice being stabilized

5.2. Using DIC (D-I-C) optics, focus on the auditory nerve root on the thick side of the slice **[1-TXT]** and use a micromanipulator to move the bipolar tungsten stimulating electrode to the auditory nerve root and gently into the surface of the tissue **[2]**.

5.2.1. SCREEN: Wide shot AN Stim MOC Target and Patch 200828: 00:05-00:19 **TEXT: DIC: digital image correlation** NOTE: Please start from specified time to orient viewers to the slice.
5.2.2. SCREEN: Wide shot AN Stim MOC Target and Patch 200828: 00:19-00:32 NOTE: Shots 5.2.1 and 5.2.2 can be played at increased speed *Video Editor: Please speed up 5.2.1 and 5.2.2)*

5.3. Move the field of view to the ventral nucleus of the trapezoid body on the thin side **[1]** and select a medial olivocochlear neuron as the target for patch clamp electrophysiology under epifluorescence using a 561-nanometer emission filter **[2]**.

5.3.1. SCREEN: Wide shot AN Stim MOC Target and Patch 200828: 00:35-00:49
5.3.2. SCREEN: 200815_151335_ChoosePatchClipped 00:00-00:12 *Video Editor: please speed up* NOTE: both 5.3.1 and 5.3.2 shots can be sped up as necessary

5.4. Fill a recording pipette with the appropriate internal solution for the proposed experiment **[1]** and, under DIC optics, patch and record from the medial olivocochlear neuron in the whole-cell configuration **[2-TXT]**.

5.4.1. Talent filling pipette
5.4.2. SCREEN: 200815_151335_ChoosePatchClipped: 00:12-00:37 *Video Editor: Speed up as necessary* **TEXT: Compensate membrane capacitance and series resistance if required**

5.5. Adjust the electrical stimulation amplitude of the auditory nerve root to obtain consistent postsynaptic events in the medial olivocochlear neuron **[1]**.

5.5.1. Stimulation amplitude being adjusted

5.6. Then run appropriate stimulation protocols to observe evoked synaptic currents in medial olivocochlear neurons **[1]**.

5.6.1. SCREEN: 200828_163058_Run Protocol_single pulse 4x speed: 00:04-00:15
NOTE: Video currently at 4X speed *Video editor: speed up as necessary*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
4.4.-4.7.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.4., 4.7.

Results

9. Results: Representative Wedge Slice Preparation and Stimulation

9.1. This wedge slice preparation is designed to contain the auditory nerve root **[1]** and the cochlear nucleus contralateral **[2]** to the medial olivocochlear neurons targeted for recordings **[3]**.

9.1.1. LAB MEDIA: Figure 1B
9.1.2. LAB MEDIA: Figure 1B *Video Editor: please add/emphasize ANR text and line*
9.1.3. LAB MEDIA: Figure 1B *Video Editor: please emphasize white dotted line*
9.1.4. LAB MEDIA: Figure 1B *Video Editor: please emphasize asterisk*

9.2. In this cresyl violet-stained, resectioned wedge slice, the cochlear nucleus is present in nearly its full rostro-caudal extent **[1]** and the auditory nerve root is observed entering the cochlear nucleus **[2]**.

9.2.1. LAB MEDIA: Figure 2
9.2.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize arrowhead in S17*

9.3. In addition, the wedge slice contains neurons of the medial nucleus of the trapezoid body ipsilateral to the medial olivocochlear neurons from which recordings are performed **[1]**.

9.3.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize dark area in S3-S15*

9.4. To confirm the neuronal connectivity within a wedge slice, presynaptic inputs are stimulated in two ways **[1]**.

9.4.1. LAB MEDIA: Figures 4A

9.5. First, the ventral acoustic stria at the midline is electrically stimulated **[1]**, activating T-stellate axons directly and the medial nucleus of the trapezoid body neurons via globular bushy cell axon stimulation and resulting in post-synaptic currents that are measured at the medial olivocochlear neuron **[2]**.

9.5.1. LAB MEDIA: Figures 4A and 4B *Video Editor: please highlight VAS Stimulation electrode in Figure 4A*
9.5.2. LAB MEDIA: Figures 4A and 4B *Video Editor: please sequentially emphasize rows or responses in Figure 4B*

9.6. The auditory nerve root is then stimulated **[1]** to activate the monaural ascending brainstem circuitry and to evoke post-synaptic responses **[2]**.

9.6.1. LAB MEDIA: Figures 4A and 4C *Video Editor: please highlight AN stimulation in Figure 4A*

9.6.2. LAB MEDIA: Figures 4A and 4C *Video Editor: please sequentially emphasize rows of responses in Figure 4C*

9.7. Comparison of the onset latency measures of the first postsynaptic current evoked with direct stimulation of the ventral acoustic stria with those evoked with auditory nerve stimulation **[1]** reveals a significantly longer latency in the auditory nerve stimulation event indicative of the synaptic delay resulting from the extra cochlear nucleus synapse **[2]**.

9.7.1. LAB MEDIA: Figure 4D

9.7.2. LAB MEDIA: Figure 4D *Video Editor: please emphasize AN data box*

Conclusion

10. Conclusion Interview Statements

10.1. **Matthew Fischl**: The use of anatomical landmarks and careful maneuvering of the magnetic disc stage are critical for creating wedge slices with intact neuronal circuitry and reliable evoked postsynaptic responses **[1]**.

10.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.4.-4.10.)

10.2. **Catherine Weisz**: This slicing technique provides a platform for using additional in vitro electrophysiology tools, including calcium or voltage imaging, optogenetics, neurotransmitter uncaging, and both intracellular and extracellular pharmacology **[1]**.

10.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera