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Title: A Head-mounted Optically Transparent Skull (HOTS) Window for Deep Transcranial Imaging of the Mouse Cortex

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? **No**
- 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? **No**
- 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: 29

Number of Shots: 47

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. **Wei Zheng:** Our research develops a transparent-skull method for noninvasive neocortical imaging, enabling long-term visualization of brain structure and function in vivo.
 - 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. **Wei Zheng:** Existing skull-imaging methods are often invasive and limited to restrained animals. Our protocol enables transparent-skull imaging in freely moving mice, improving transparency and extending observation time.
 - 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. **Hui Li:** The protocol enables transcranial two-photon imaging of deep cortical structures and neuronal calcium dynamics in adult mice.
 - 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. **Lina Liu:** The most important consideration is positioning the HOTS window away from cranial sutures to ensure optimal transparency, stability, and imaging quality.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Hui Li:** After HOTS preparation, researchers can combine transcranial imaging with optogenetic, photopharmacological, or other light-based manipulations.

- 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 the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences

Protocol

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

2. Skull Preparation and Headplate Fixation

Demonstrator: Lina Liu

- 2.1. After preparing the anesthetized animal for the surgery [1-TXT], use fine forceps and surgical scissors to remove the scalp over the entire dorsal skull surface [2].
 - 2.1.1. Establishing shot of the talent with the anesthetized animal placed on the surgical platform. **TXT: Anesthesia: 1% (w/v) Sodium Pentobarbital**
***Videographer:** Please obtain CU shot as well for this shot*
 - 2.1.2. Talent removing the scalp over the dorsal skull surface with fine forceps and surgical scissors.
- 2.2. Using fine forceps, gently remove the periosteum from the skull surface [1].
 - 2.2.1. Talent removing the periosteum from the skull surface with fine forceps.
- 2.3. Then, clean the surface with a moist cotton-tipped applicator [1] and dry the skull with a gentle stream of air [2].
 - 2.3.1. Talent cleaning the skull surface with a moist cotton-tipped applicator.
 - 2.3.2. Talent drying the skull with a gentle stream of air.
- 2.4. Next, prepare the dental cement according to the manufacturer's instructions [1]. Apply a thin layer of the cement to the exposed skull surface and surrounding tissues for protection [2] while leaving the region designated for the clearing window uncoated [3].
 - 2.4.1. Talent preparing the dental cement.
 - 2.4.2. Talent applying a thin layer of dental cement to the exposed skull surface and surrounding tissues.
 - 2.4.3. Shot of the region designated for the clearing window free of dental cement.
- 2.5. Using dental cement, secure a small titanium headplate to the skull [1].
 - 2.5.1. Talent securing a titanium headplate to the skull with dental cement.

3. Creating a Head-mounted Optically Transparent Skull (HOTS) Window

- 3.1. Once the dental cement cures for about 5 minutes, cover it with a thin layer of self-curing dental soft liner, excluding the clear window region, to create a continuous seal

over the skull [1].

3.1.1. Talent applying a thin layer of self-curing dental soft liner material over the cured dental cement while leaving the clearing window region uncovered.

3.2. Then, shape the self-curing dental soft-liner material smoothly around the planned clearing window margin, creating a flat sealing surface for the cap [1].

3.2.1. Talent shaping the self-curing dental soft-liner material around the planned clearing window margin, forming a flat sealing surface for the cap.

3.3. Cut the head of a disposable plastic pipette to create a small cylindrical cap with a closed top and open bottom [1]. Place the cap over the designated clearing region and press gently into the soft-liner material [2].

3.3.1. Talent cutting the head of a disposable polyethylene plastic pipette to create a small cylindrical cap with a closed top and open bottom.

3.3.2. Talent places the cap over the designated clearing window region and presses it.

3.4. Fix the cap in place using additional soft-liner material around its base [1]. Ensure that the junction between the cap and skull is fully sealed [2].

3.4.1. Talent applying additional soft liner material around the base of the cap.

3.4.2. Shot of the fully sealed junction between the cap and skull.

3.5. Using a 1 milliliter syringe with a 26-gauge needle, pierce one or more small holes in the cap wall to create ports for injecting and withdrawing clearing solutions [1].

3.5.1. Talent piercing small holes in the cap wall with a 1 milliliter syringe fitted with a 26 gauge needle.

3.6. Allow the soft-liner material to cure completely and ensure that the cap is securely attached, with no visible gaps at the interface [1].

3.6.1. Talent checking that the cap is firmly attached with no visible gaps.

3.7. Then, remove the mouse from the stereotaxic frame [1] and place it in a warm cage until full recovery from anesthesia [2].

3.7.1. Talent removing the mouse from the stereotaxic frame.

3.7.2. Talent placing the mouse in a warm cage for recovery from anesthesia.

4. Skull Clearing in Awake, Freely Behaving Mice

4.1. After anesthetizing the mouse briefly, gently restrain it [1-TXT] and stabilize the head-mounted cap by hand [2].

4.1.1. Talent gently restraining the mouse. **TXT: Anesthesia: 3-4% Isoflurane**

- 4.1.2. Talent stabilizing the head-mounted cap by hand.
- 4.2. Using a 1 milliliter syringe with a 26-gauge needle, slowly inject approximately 1 milliliter of S1 into the head-mounted cap [1].
 - 4.2.1. Talent injecting S1 into the head-mounted cap with a 1 milliliter syringe fitted with a 26 gauge needle.
- 4.3. Return the mouse to its home cage and allow S1 to act on the skull for 2 hours [1].
 - 4.3.1. Talent placing the mouse in a home cage.
- 4.4. After a brief anesthesia, aspirate S1 from the cap and refill it with fresh S1 [1]. Then, return the mouse to its cage and allow S1 to act for another 2 hours. [1].
 - 4.4.1. Talent aspirating S1 from the head-mounted cap and refilling it.
 - 4.4.2. Talent placing the mouse back into the cage.
- 4.5. Repeat the procedure again and replace S1 with 0.7 milliliters of S2 after 6 hours of S1 treatment [1].
 - 4.5.1. Talent aspirating S1 from the head-mounted cap and 0.7 milliliters of S2 with a 1 milliliter syringe.
- 4.6. Then, return the mouse to the cage and allow S2 to act on the skull for 1 hour to further enhance transparency and provide refractive index matching [1].
 - 4.6.1. Talent placing the mouse back into the cage following S2 administration.

5. Head Fixation for Imaging

- 5.1. After anesthetizing the mouse at the end of the S2 incubation [1-TXT], confirm adequate anesthesia using the toe-pinch reflex test [2].
 - 5.1.1. Shot of the anesthetized mouse placed at the surgical platform. **TXT: Anesthesia: 1% Sodium Pentobarbital (150–200 μ L/20 g of body weight)**
 - 5.1.2. Talent performing the toe-pinch reflex test on the mouse.
- 5.2. To fix the mouse's head, screw the titanium headplate to a custom-designed head holder [1]. Apply ophthalmic ointment to both eyes to prevent drying during imaging [2].
 - 5.2.1. Talent securing the titanium headplate to the custom-designed head holder.
 - 5.2.2. Talent applying ophthalmic ointment to both eyes of the mouse.
- 5.3. To expose the cleared skull surface, using a syringe, aspirate the S2 solution from the cap [1].
 - 5.3.1. Talent aspirating the S2 solution from the cap with a syringe.
- 5.4. Using fine scissors, carefully cut the plastic cap [1] and remove it with forceps, taking

care not to apply excessive force on the skull [2].

5.4.1. Talent cutting the plastic cap with fine scissors.

5.4.2. Talent removing the plastic cap with forceps.

5.5. Then, gently peel off the soft-liner material surrounding the clearing window to fully expose the cleared skull region [1].

5.5.1. Talent peeling off the soft liner material surrounding the clearing window.

5.6. Apply a small drop of S2 solution directly onto the cleared skull to serve as a refractive index-matching immersion medium during imaging [1].

5.6.1. Talent applying a small drop of S2 solution onto the cleared skull surface.

6. Post-Imaging Skull Restoration and Animal Recovery

6.1. Gently aspirate and remove any residual S2 solution from the skull surface [1]. Rinse the cleared skull region with PBS several times to promote gradual restoration of the skull's native opaque state [2].

6.1.1. Talent aspirating residual S2 solution from the skull surface.

6.1.2. Talent rinsing the cleared skull region with phosphate-buffered saline.

6.2. Using sterile cotton swabs, gently dry residual fluid from the skull and surrounding tissue while avoiding mechanical damage to the bone [1].

6.2.1. Talent drying residual fluid from the skull and surrounding tissue with sterile cotton swabs.

6.3. If the animal is kept alive for subsequent experiments, apply a layer of Kwik-Cast over the skull window to protect the bone during the survival period [1]. Then, allow the protective layer to cure completely [1].

6.3.1. Talent applying a layer of Kwik-Cast over the skull window.

6.3.2. Talent placing the preparation aside to allow the protective layer to fully cure.

6.4. Detach the mouse from the head holder [1] and place it in a warm cage [2].

6.4.1. Talent detaching the mouse from the head holder.

6.4.2. Talent placing the mouse in a warm cage.

Results

7. Results

- 7.1. Ex vivo imaging of skull pieces placed on a resolution target showed a stepwise increase in transparency after S1 [1] and S2 treatment [2]. In vivo bright-field imaging revealed pial vasculature through the cleared skull [3].
 - 7.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the image labeled “S1”*
 - 7.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the image labeled “S2”*
 - 7.1.3. LAB MEDIA: Figure 2B.
- 7.2. Two-photon imaging through the HOTS (*Hots*) window visualized neuronal structures [1].
 - 7.2.1. LAB MEDIA: Figure 2C–F.
- 7.3. The HOTS window enabled maximum imaging depth approaching 800 micrometers below the pia [1].
 - 7.3.1. LAB MEDIA: Figure 2F. *Video editor: Highlight the taller bar labeled “After” reaching close to 800 micrometers.*
- 7.4. A direct before-and-after comparison of the same cortical region revealed that neurites appeared blurred at approximately 100 to 200 micrometers through the native skull [1], while fine structures were distinctly visible at the same depth after clearing [2].
 - 7.4.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the images in the “Before” row at 150 and 200 micrometers showing faint and blurred structures.*
 - 7.4.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the images in the “After” row at 150 and 200 micrometers showing clearly visible fine structures.*
- 7.5. Neuronal somata remained identifiable at 500 to 650 micrometers after clearing [1].
 - 7.5.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the images labelled 500 and 650 micrometers in the “After” row.*
- 7.6. In awake Thy1-GCaMP6s (*Thy-One-G-Camp-Six-S*) mice, two-photon imaging at 150 micrometers depth in the barrel cortex captured spontaneous calcium activity from multiple neurons through the cleared skull [1].
 - 7.6.1. LAB MEDIA: Figure 3A and B.
- 7.7. F/F0 (*F-Over-F-naught*) traces of marked somata revealed heterogeneous activity patterns across cells [1].
 - 7.7.1. LAB MEDIA: Figure 3C.

1. Sodium Pentobarbital
Pronunciation link: <https://www.merriam-webster.com/medical/pentobarbital>
IPA: /'soʊ.di.əm ,pen.too'ba:r.bi.təl/
Phonetic Spelling: soh·dee·uhm pen·toh·bar·bih·tuhl
2. Periosteum
Pronunciation link: <https://www.merriam-webster.com/dictionary/periosteum>
IPA: /,per.i'ɑ:.sti.əm/
Phonetic Spelling: pehr·ee·os·tee·uhm
3. Titanium
Pronunciation link: <https://www.merriam-webster.com/dictionary/titanium>
IPA: /taɪ'teɪ.ni.əm/
Phonetic Spelling: ty·tay·nee·uhm
4. Optically
Pronunciation link: <https://www.merriam-webster.com/dictionary/optical>
IPA: /'ɑ:p.tɪ.kəl.i/
Phonetic Spelling: op·tih·kuh·lee
5. Polyethylene
Pronunciation link: <https://www.merriam-webster.com/dictionary/polyethylene>
IPA: /,pɑ:..li'εθ.ə.li:n/
Phonetic Spelling: pol·ee·eth·uh·leen
6. Stereotaxic
Pronunciation link: <https://www.merriam-webster.com/dictionary/stereotaxic>
IPA: /,ster.i.oo'tæk.sɪk/
Phonetic Spelling: stair·ee·oh·tak·sik
7. Isoflurane
Pronunciation link: <https://www.merriam-webster.com/medical/isoflurane>
IPA: /,aɪ.sou'flʊr.əm/
Phonetic Spelling: eye·soh·floor·ayn
8. Refractive
Pronunciation link: <https://www.merriam-webster.com/dictionary/refractive>
IPA: /rɪ'fræk.tɪv/
Phonetic Spelling: ree·frak·tiv
9. Ophthalmic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ophthalmic>
IPA: /ɑ:f'θæl.mɪk/
Phonetic Spelling: off·thal·mik
10. Phosphate-buffered saline
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>
IPA: /'fɑ:s.feɪt 'bʌf.əd sə'li:n/
Phonetic Spelling: fos·fayt buf·erd suh·leen
11. Vasculature
Pronunciation link: <https://www.merriam-webster.com/dictionary/vasculature>
IPA: /'væs.kjə.lə.tʃə/
Phonetic Spelling: vas·kyuh·luh·cher

12. Two-photon
Pronunciation link: <https://www.merriam-webster.com/dictionary/photon>
IPA: /ˌtuː ˈfoʊ.tən/
Phonetic Spelling: too·foh·ton
13. Neuronal
Pronunciation link: <https://www.merriam-webster.com/dictionary/neuronal>
IPA: /nʊˈraː.nəl/
Phonetic Spelling: noor·on·uhl
14. Cortical
Pronunciation link: <https://www.merriam-webster.com/dictionary/cortical>
IPA: /ˈkɔːr.təl/
Phonetic Spelling: kor·tuh·kuhl
15. Neurites
Pronunciation link: <https://www.merriam-webster.com/medical/neurite>
IPA: /ˈnɔr.aɪt/
Phonetic Spelling: noor·yte
16. Thy1-GCaMP6s
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: thy·one gee·kamp·six·ess
17. Somata
Pronunciation link: <https://www.merriam-webster.com/dictionary/soma>
IPA: /ˈsoʊ.mə.tə/
Phonetic Spelling: soh·muh·tuh
18. Heterogeneous
Pronunciation link: <https://www.merriam-webster.com/dictionary/heterogeneous>
IPA: /ˌhet̬.ə.əˈdʒiː.ni.əs/
Phonetic Spelling: het·uh·ruh·jee·nee·uhs
19. Calcium
Pronunciation link: <https://www.merriam-webster.com/dictionary/calcium>
IPA: /ˈkæl.si.əm/
Phonetic Spelling: kal·see·uhm
20. Transparency
Pronunciation link: <https://www.merriam-webster.com/dictionary/transparency>
IPA: /trænˈsper.ən.si/
Phonetic Spelling: trans·pair·uhn·see