

Submission ID #: 69589

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Title: Intravital Two-Photon Imaging of Touch Sensory Axon Morphology in Mouse Skin

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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? **Yes, all done**
- 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: 19

Number of Shots: 42 (8 SC)

Introduction

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

INTRODUCTION:

- 1.1. **Shan Meltzer:** We are investigating mammalian somatosensory circuit formation in the skin through in vivo imaging of sensory axon development and regeneration.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.
- 1.2. **Shan Meltzer:** Recent developments in mouse genetic tools for somatosensation research allow us to study sensory axon development in vivo with subtype specificity.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

- 1.3. **Shan Meltzer:** Our protocol addresses a challenge in the field to visualize developing sensory axon structure and development in vivo.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.
- 1.4. **Shan Meltzer:** Our technique enables high-resolution, consistent, and chronic imaging of LTMRs during development and in adult mice *in vivo*.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.5. **Shan Meltzer:** This study will enable the investigation of the in vivo mechanisms and dynamic processes behind touch-circuit development and regeneration in the future.

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.

Videographer's NOTE: Sydney Kong is no longer at Vanderbilt, so there is no headshot for her. .

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at the Vanderbilt University

Protocol

2. Mouse Paw Preparation and Tattooing

Demonstrator: Emily Barnes

2.1. To begin, turn on the laser and allow sufficient time for it to warm up [1].

2.1.1. WIDE: Talent turning on the laser.

2.2. Then, turn on the two-photon microscope [1] and open the control and acquisition software on the connected computer [2].

2.2.1. Talent switching on the two-photon microscope.

2.2.2. Show the talent opening the **control software**.

2.3. Turn on the heating pad and set the temperature to 37 degrees Celsius to prevent hypothermia during anesthesia [1].

2.3.1. Talent switching on the heating pad and adjusting the temperature setting to 37 degrees Celsius.

2.4. Now, transfer the anesthetized mouse to the heating pad on the imaging platform [1-TXT].

2.4.1. Talent positioning the anesthetized mouse on the heating pad atop the imaging platform. **TXT: Anesthesia: Isoflurane**

Induction: 3.5%

Maintenance: 1.5% for adult mouse and 2 - 4% for mice younger than P20

2.5. Align the mouse body on a platform adjacent to the imaging stage so that it does not touch the area where the paw is mounted, minimizing movement artifacts during imaging [1]. Now, apply ophthalmic ointment to both eyes of the mouse to prevent dryness during imaging [2].

2.5.1. Talent adjusting the mouse position so the body rests on a separate platform next to the imaging stage.

2.5.2. Talent applying ophthalmic ointment to the eyes using a sterile applicator.

- 2.6. Next, using a cotton swab, gently apply depilatory cream to the mouse paw to remove hair [1]. After one minute, remove the cream [2] and clean the paw using 70 percent ethanol followed by water [3].
 - 2.6.1. Talent applying depilatory cream with a cotton swab to the mouse paw.
 - 2.6.2. Talent removing the cream by wiping.
 - 2.6.3. Talent wiping the paw with ethanol, then water.
- 2.7. Then, with one hand, gently stretch the skin to stabilize the paw surface [1]. Using a sterile 30-gauge needle and black ink, pierce the skin and tattoo two adjacent dots on either side of the target imaging region, spaced approximately 2 millimeters apart [2]. After tattooing, dispose of the used needle in a sharps container [3].
 - 2.7.1. Talent stabilizing the paw skin by stretching it gently with one hand.
 - 2.7.2. Talent using a sterile 30 gauge needle dipped in black ink to tattoo two dots on the skin, spaced 2 millimeters apart.
 - 2.7.3. Talent placing the used needle into a sharps disposal container.

3. Slide Assembly and Paw Mounting for Imaging

- 3.1. Take a glass microscope slide [1] and place four dots of vacuum grease at the corners [2]. Then, position a 1-millimeter-thick piece of modeling clay at the center of the slide [3]. Place the slide under the objective lens and center the mouse's paw directly on top of the clay [3].
 - 3.1.1. Talent placing a glass slide on the work surface.
 - 3.1.2. Talent applying four small dots of vacuum grease at the corners of the microscope slide.
 - 3.1.3. Talent placing a thin, flat piece of modeling clay approximately 1 millimeter thick in the center of the slide.
 - 3.1.4. Talent positioning the slide under the objective lens and aligning the mouse's paw over the clay.
- 3.2. Gently roll the mouse's paw on the slide to flatten it and press it into the clay [1]. Then, carefully place a 22 by 60-millimeter coverslip over the paw to secure it [2]. Adjust the angle of the mouse skin by applying light pressure on each corner of the coverslip until the skin surface is parallel to the imaging plane [3-TXT].

- 3.2.1. Talent rolling the mouse's paw gently to flatten and embed it into the clay.
- 3.2.2. Talent placing a 22 by 60 millimeter coverslip on top of the paw.
- 3.2.3. Talent pressing lightly on each corner of the coverslip to align the skin surface parallel to the imaging plane. **TEXT: Perform this step gently to avoid injury**

3.3. To image the glabrous skin, use tweezers to rotate the paw so the glabrous skin faces upward [1] and then use the flat end of the tweezers to gently press the paw into the clay [2]. Once flattened, place the coverslip on top and press down carefully [3]. Confirm that the paw is centered [4].

3.3.2, Talent using tweezers to rotate the paw until the glabrous skin is facing up.

3.3.3, Talent pressing the paw into the clay with the flat end of the tweezers.

3.3.4, Talent placing the coverslip over the paw and gently pressing down.

3.3.1, View of the aligned paw displayed on the LED screen. **NOTE: Move this shot (3.3.1) below shot 3.3.4. Vo adjusted**

4. Two-Photon Microscope Imaging of Low-Threshold Mechanoreceptor (LTMR) Morphology

4.1. Place a drop of distilled water on the coverslip directly above the mouse paw [1].

4.1.1. Talent using a pipette to carefully place a drop of distilled water on the coverslip, directly over the mouse paw. **Videographer's NOTE: Shot 4-1-1 was mis-slated as 4-1-4, but the file name is correct.**

4.2. Lower the objective lens until a water column forms between the lens and the coverslip [1].

4.2.1. Close-up of slowly lowering the objective lens.

4.3. Once the objective lens is centered over the mouse paw, close the microscope box [1] and use the LED screen to focus on the sample by adjusting the X, Y, and Z controllers [2]. The LED screen will show a live image illuminated by the microscope's built-in LED [3].

4.3.1. Talent closing the microscope enclosure.

4.3.2. Talent turns on the LED screen and adjusting the X, Y, and Z knobs while watching the screen to focus on the sample.

- 4.3.3. Show the LED screen displaying the live illuminated image of the sample as the talent fine-tunes the focus.
- 4.4. After locating the sample, turn off the LED screen [1]. To begin two-photon imaging, flip the lever on the upper-right side of the microscope to switch from the mirror setting to the dichroic setting [2].
- 4.4.1. Talent turning off the LED screen using the designated button.
- 4.4.2. Talent flipping the lever on the microscope to switch from mirror to dichroic setting.
- 4.5. Then, close the blackout curtains [1] and switch on the detector to visualize yellow fluorescent protein or YFP [2].
- 4.5.1. Talent pulling blackout curtains shut around the imaging area.
- 4.5.2. Talent activating the detector for YFP visualization. **Videographer's NOTE:** Shot 4-5-2 has no slate, as it was shot in a blacked out room, as required by the equipment in use. (Image is of the lighted display of the unit)
- 4.6. In Prairie View, open the **shutter** and switch the software to imaging mode [1]. For imaging Thy1-YFP (*thigh-1-Y-F-P*), turn on the tunable laser and set the wavelength to 960 nanometers with a gain between 700 and 800 [2]. For green fluorescent protein or GFP imaging, adjust the laser to 920 nanometers [3].
- 4.6.1. SCREEN: 4.6.1.
- 4.6.2. SCREEN: 4.6.2 00:00-00:08 and 00:15-00:23
- 4.6.3. SCREEN: 4.6.3 00:00-00:15 .
- 4.7. For mice aged postnatal day 10 or younger, set the initial laser power to 50 [1] and gradually increase the power until the desired signal intensity is achieved [2]. This minimizes light penetration and reduces the risk of photodamage. Once the laser wavelength, gain, and power are set, begin scanning the tissue [3].
- 4.7.1. SCREEN: 4.7.1.
- 4.7.2. SCREEN: 4.7.2.
- 4.7.3. SCREEN: 4.7.3 00:15-00:25

4.8. Now, use the X, Y, and Z controllers to locate the imaging field and adjust the zoom as necessary [1]. Start image acquisition at a position approximately 10 to 20 micrometers beneath the skin surface [2].

4.8.1. Talent adjusting the X, Y, and Z knobs to identify the appropriate field and modify zoom settings.

4.8.2. SCREEN: 4.8.2.

4.9. Finally, remove the mouse paw from the glass slide after imaging [1] and return the mouse to a recovery cage for postoperative care [2].

4.9.1. Talent gently raises the objective, removes the coverslip, and lifts the mouse paw from the glass slide.

4.9.2. Talent transferring the mouse to a recovery cage and observing its condition.

Results

5. Results

5.1. In the hairy skin of adult mice, the overall axonal terminal organization in the forepaw was revealed through a maximum intensity projection, showing fully developed lanceolate endings surrounding hair follicles [1]. The average density of lanceolate endings surrounding hair follicles in these mice was 15.8 endings per square millimeter [2].

5.1.1. LAB MEDIA: Figure 2A.

5.1.2. LAB MEDIA: Figure 2B.

5.2. A sparse, crescent-shaped A δ (A-delta) -LTMR lanceolate ending was observed innervating a hair follicle at postnatal day 4 [1-TXT].

5.2.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the crescent-shaped ending near the red arrowhead.* **TXT: LTMR: Low-Threshold Mechanoreceptor**

5.3. A longitudinal lanceolate complex in hairy skin was imaged in an adult mouse at postnatal day 39 [1] and high-resolution z-plane (zee-plane) images showed individual A β (A-beta) rapidly adapting-LTMR lanceolate endings, each separated by 3 micrometers [2].

5.3.1. LAB MEDIA: Figure 2D. *Video editor: Show the close-up image labeled P39 showing the structure of the lanceolate complex.*

5.3.2. LAB MEDIA: Figure 2E. *Video editor: Highlight each of the three sequential z-plane images labeled P39 and highlight the "3 μ m" separation between images.*

5.4. Immature lanceolate complexes were observed in the hairy skin at postnatal day 10, with fewer A β -LTMR axons [1] and individual z-planes revealed sparse nascent lanceolate endings [2].

5.4.1. LAB MEDIA: Figure 2F.

5.4.2. LAB MEDIA: Figure 2G.

5.5. In the adult mouse fingertip, A β RA-LTMR axons were visualized innervating Meissner corpuscles as ellipsoid structures beneath the epidermis [1] and individual optical sections highlight discrete axon terminals at different depths within the skin [2].

- 5.5.1. LAB MEDIA: Figure 2H. *Video editor: Show the wide field image of the fingertip labeled P52 with Meissner corpuscle structures.*
 - 5.5.2. LAB MEDIA: Figure 2I. *Video editor: Highlight the three separate z-stack frames sequentially.*
 - 5.6. Across three consecutive days, the same nerve bundles in adult mouse hairy skin were identified, with slight shape variations likely due to paw positioning [1].
 - 5.6.1. LAB MEDIA: Figure 3A B C. *Video editor: Highlight the red arrows pointing to nerve bundles in all 3 images.*
 - 5.7. The same axonal morphologies around hair follicles in the hairy skin were consistently imaged over 3 days [1].
 - 5.7.1. LAB MEDIA: Figure 3D E F.
-
- 1. Two-photon
Pronunciation link: <https://www.merriam-webster.com/dictionary/two-photon>
IPA: /'tu: ˌfoʊ ˌtɑ:n/
Phonetic Spelling: too·foh·ton
 - 2. Hypothermia
Pronunciation link: <https://www.merriam-webster.com/dictionary/hypothermia>
IPA: / ˌhaɪ.poo'θɜ:.mi.ə/
Phonetic Spelling: hy·poh·thur·mee·uh
 - 3. Anesthesia
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthesia>
IPA: / ˌæn.əs'θi:.ʒə/
Phonetic Spelling: an·uhs·thee·zhuh
 - 4. Isoflurane
Pronunciation link: <https://www.merriam-webster.com/dictionary/isoflurane>
IPA: / ˌaɪ.sou'flɒr.eɪn/
Phonetic Spelling: eye·soh·floor·ayn
 - 5. Ophthalmic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ophthalmic>
IPA: /ɑ:f'θæl.mɪk/
Phonetic Spelling: off·thal·mik

6. Depilatory
Pronunciation link: <https://www.merriam-webster.com/dictionary/depilatory>
IPA: /dɪˈpɪl.əˌtɔːr.i/
Phonetic Spelling: dih·pill·uh·tor·ee
7. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /ˈɛθ.ə.næl/
Phonetic Spelling: eth·uh·nahl
8. Glabrous
Pronunciation link: <https://www.merriam-webster.com/dictionary/glabrous>
IPA: /ˈgleɪ.brəs/
Phonetic Spelling: glay·bruhs
9. Dichroic
Pronunciation link: <https://www.merriam-webster.com/dictionary/dichroic>
IPA: /daɪˈkroʊ.ɪk/
Phonetic Spelling: dye·kroh·ik
10. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flʊˈres.ənt/
Phonetic Spelling: floor·ess·uhnt
11. Tunable
Pronunciation link: <https://www.merriam-webster.com/dictionary/tunable>
IPA: /ˈtuː.nə.bəl/
Phonetic Spelling: too·nuh·buhl
12. Nanometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>
IPA: /ˈnæn.oo.miː.tə/
Phonetic Spelling: nan·oh·mee·ter
13. Photodamage
Pronunciation link: <https://www.merriam-webster.com/dictionary/photodamage>
IPA: /ˌfoʊ.touˈdæm.ɪdʒ/
Phonetic Spelling: foh·toh·dam·ij
14. Micrometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkroʊ.miː.tə/
Phonetic Spelling: my·kroh·mee·ter
15. Mechanoreceptor
Pronunciation link: <https://www.merriam-webster.com/dictionary/mechanoreceptor>
IPA: /ˌmek.ə.noʊ.rɪˈsep.tə/
Phonetic Spelling: meh·kuh·noh·rih·sep·ter
16. Axonal
Pronunciation link: <https://www.merriam-webster.com/dictionary/axonal>
IPA: /ˈæk.sə.nəl/
Phonetic Spelling: ak·suh·nuhl

17. Lanceolate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lanceolate>
IPA: /'læn.si.ə.leɪt/
Phonetic Spelling: lan·see·uh·layt
18. Follicles
Pronunciation link: <https://www.merriam-webster.com/dictionary/follicle>
IPA: /'fɑː.li.kəl/
Phonetic Spelling: fah·luh·kuhl
19. Postnatal
Pronunciation link: <https://www.merriam-webster.com/dictionary/postnatal>
IPA: /,poust'nei.təl/
Phonetic Spelling: pohst·nay·tuhl
20. Epidermis
Pronunciation link: <https://www.merriam-webster.com/dictionary/epidermis>
IPA: /,ɛp.ə'dɜː.mɪs/
Phonetic Spelling: eh·puh·dur·miss
21. Ellipsoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/ellipsoid>
IPA: /ɪ'lip.sɔɪd/
Phonetic Spelling: ih·lip·soyd
22. Meissner
Pronunciation link: <https://www.merriam-webster.com/dictionary/Meissner>
IPA: /'maɪs.nə/
Phonetic Spelling: myce·ner
23. Corpuscles
Pronunciation link: <https://www.merriam-webster.com/dictionary/corpuscle>
IPA: /'kɔːr.pʌ.səl/
Phonetic Spelling: kor·puh·suhl