Submission ID #: 68284
Scriptwriter Name: Debopriya Sadhukhan
Project Page Link: https://review.jove.com/account/file-uploader?src=20826668

Title: In Vivo Calcium Imaging of Neuronal Ensembles in Networks of Primary Sensory Neurons in Intact Trigeminal Ganglia

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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**

   Can you record movies/images using your own microscope camera?

   No

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 17
Number of Shots: 49

Introduction

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

REQUIRED:

1.1. **John Shannonhouse:** This research focuses on peripheral ganglia neural networks, aiming to understand the signals and intercellular interactions involved in pain, itch, and touch sensation.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1, 3.2.2.*

What are the current experimental challenges?

1.2. **John Shannonhouse:** Neurons sense stimuli, but studying their activity in vivo under normal physiological conditions remains highly challenging.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4B, 4C, 4E, 4F.*

What significant findings have you established in your field?

1.3. **John Shannonhouse:** Our research highlights the importance of Mrgprb2 receptor in both alcohol withdrawal and stress-induced pain.

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

What research gap are you addressing with your protocol?

1.4. **Eungyung Kim:** Our protocol allows the study of trigeminal ganglion neuron activation at the population level in direct response to stimuli, which is physiologically important but technically very challenging.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4A.*

How will your findings advance research in your field?

1.5. **Eungyung Kim:** Data produced by this protocol serves as a powerful complement to behavior, cell culture, and immunohistochemistry data, allowing investigation of the immediate effects of stimuli or drugs on an entire ganglion.

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.

Testimonial Questions:

Videographer: Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **John Shannonhouse**: The technical difficulty of trigeminal in vivo imaging remains a major obstacle to its wider use. We hope more people will adopt this method and recognize its value. They can help discover new ways to use it.

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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE?

1.7. **John Shannonhouse**: We get tremendous value out of collaborations. We can perform in vivo imaging with other investigators' pain models. We hope more investigators will want to collaborate with us.

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

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio

Protocol

2. Surgical Procedure for the Right-Side Trigeminal Ganglion Imaging

Demonstrator: Eungyung Kim

2.1. To begin, place the anesthetized mouse on a heating pad to maintain the body temperature at 37 degrees Celsius **[1-TXT]**, then position the head in a stereotactic mask with a tilt of approximately 15 degrees to the left **[2]**. Apply ophthalmic ointment to the eyes to prevent dryness and irritation **[4]**.

2.1.1. WIDE: Talent placing the anesthetized mouse on the heating pad. **TXT: Anesthesia: 2.25 µL/g ketamine/xylazine i.p. per g of body weight (90 mg/kg ketamine, 13.5 mg/kg xylazine)**

2.1.2. Talent adjusting the stereotactic mask with the mouse's head tilted 15 degrees to the left.

2.1.3. Talent applying ophthalmic ointment to the mouse's eyes using a gloved finger or applicator.

2.2. After shaving the right side of the mouse's head **[1]**, make a rectangular incision of 9 millimeters by 5 millimeters between the right ear and the right eye **[2-TXT]**. Cut the tissue on the skull surface just ventral to the eye **[3]**. Stop any bleeding using a hemostatic swab or a laboratory wipe **[4]**.

2.2.1. A shot of the shaved right side of the mouse's head.

2.2.2. Talent making a 9 millimeter by 5 millimeter incision between the right ear and the right eye. **TXT: Pinch the hindpaw to confirm surgical anesthetic plane before incision**

2.2.3. Talent cutting tissue on the skull surface just ventral to the eye.

2.2.4. Talent dabbing the incision with a hemostatic swab to stop the bleeding.

2.3. Using a dental drill and a taper fissure bur, drill a hole approximately 9 millimeters by 5 millimeters in the dorsal skull centered on the incision site **[1-TXT]**. Tilt the head until the trigeminal ganglion is clearly visible **[2]**. If there is bleeding on the trigeminal ganglion, aspirate the blood or remove it using a hemostatic swab or laboratory wipe **[4]**. Ensure the trigeminal ganglion is clearly visible without removing any cortical tissue **[5]**.

2.3.1. Talent drilling a 9 millimeter by 5 millimeter hole on the skull using the dental drill with taper fissure bur. **TXT: Apply the bur as lightly as possible to the skull**

surface

2.3.2. Talent tilting the head of the stereotactic frame to visualize the trigeminal ganglion.

2.3.3. Talent aspirating blood on the trigeminal ganglion with a hemostatic swab or laboratory wipe.

2.3.4. A shot of the clearly visible trigeminal ganglion.

2.4. Move the animal and heating pad to the microscope stage **[1-TXT]**. After confirming the mouse receives continuous isoflurane anesthesia, position the stereotaxic frame so that the objective of the microscope is 9 millimeters directly above the cranial opening **[2]**. Insert the rectal thermometer **[4]** and connect the power cord to the thermometer and heating pad **[5]**.

2.4.1. Talent placing the mouse and heating pad onto the microscope stage. **TXT: Position the mouse's nose in the stereotaxic cone; Connect 1 – 1.5% isoflurane/O_2 lines for continuous delivery**

2.4.2. Talent positioning the stereotaxic frame to align the microscope objective 9 millimeters above the cranial window.

2.4.3. Talent inserting the rectal thermometer gently.

2.4.4. Talent plugging in the thermometer and heating pad.

3. Trigeminal Ganglion (TG) Imaging

Demonstrator: John Shannonhouse

3.1. Use a 5x *(five-ex)* objective to locate the surface of the trigeminal ganglion with the microscope **[1-TXT]**. Adjust the objective and the nose cone to flatten the ganglion surface and maximize the surface area in the focal plane **[2]**. Now, load the given microscope high-speed scanning protocol to scan the trigeminal ganglion in short bursts of 6 to 8 cycles **[3]**. NOTE: The VO has been edited.

3.1.1. SCOPE: The surface of the trigeminal ganglion being located with the microscope. **TXT: Objective lens: 5x/0.25 M27**

3.1.2. Talent adjusting both objective and stereotaxic nose cone to flatten the imaging surface.

3.1.3. TEXT on PLAIN BACKGROUND:

Microscope high-speed scanning protocol:

- Voxel size 4.160 µm x 4.160 µm x 14 µm
- 512 x 512 pixels
- 10 optical slices Z-stack
- 1.02 airy units (AU)/33 µm
- 15% 488 nm laser power/75 mW
- Pixel time: 1.52 µs
- Line time: 0.91 ms
- Frame time: 465 ms
- LSM scan speed: 8
- Bidirectional scanning
- GaAsP-PMT detector gain: 550 V
- Digital gain: 1

~~3.2. Scan the trigeminal ganglion in short bursts of 6 to 8 cycles **[1]**. Apply orthogonal projections of the scans to create a movie and check the image clarity and consistency between frames **[2 TXT]**.~~ NOTE: The authors requested to skip these shots.

~~3.2.1. SCREEN: To be provided by authors: Scanning the trigeminal ganglion in short bursts of 6 to 8 cycles.~~

~~3.2.2. SCREEN: To be provided by authors: Orthogonal projections of the scans being applied to create a movie, and the image clarity and consistency between frames being checked. **TXT: Adjust the frame position and section thickness if needed until the movie is clear and consistent**~~

3.3. Load the microscope high-resolution scanning protocol to create a high-resolution image of the trigeminal ganglion **[1]**. NOTE: The VO has been edited.

3.3.1. TEXT on PLAIN BACKGROUND:

Microscope high-speed scanning protocol to create a high-resolution image of TG:

- Voxel size: 0.520 µm x 0.520 µm x 14 µm
- 4096 x 4096 pixels
- 6 optical slice Z-stack
- 1.02 airy unit (AU)/33 µm

- 20% 488 nm laser power/100 mW
- Pixel time: 0.52 µs
- Line time: 4.95 ms
- Frame time: 20.26 s
- LSM scan speed: 6
- Bidirectional scanning
- GaAsP-PMT detector gain: 550 V
- Digital gain: 1

~~3.3.2. SCREEN: To be provided by authors: A high-resolution image of the trigeminal ganglion captured by the microscope.~~ NOTE: The authors requested to skip this shot.

3.4. Again, load the high-speed scanning protocol used to create the orthogonal projection movie **[1]**. NOTE: The VO has been edited.

3.4.1. TEXT on PLAIN BACKGROUND:

Microscope high-speed scanning protocol to create an orthogonal projection movie:

- Voxel size 4.160 µm x 4.160 µm x 14 µm
- 512 x 512 pixels
- 10 optical slices Z-stack
- 1.02 airy units (AU)/33 µm
- 15% 488 nm laser power/75 mW
- Pixel time: 1.52 µs
- Line time: 0.91 ms
- Frame time: 465 ms
- LSM scan speed: 8
- Bidirectional scanning
- GaAsP-PMT detector gain: 550 V
- Digital gain: 1

~~3.4.2. SCREEN: To be provided by authors: Spontaneous activity being recorded for 80 cycles.~~NOTE: The authors requested to skip these shots.

~~3.4.3. SCREEN: To be provided by authors: The movie being created.~~

~~3.4.4. SCREEN: To be provided by authors: Verifying that the images are clear and consistent enough for analysis~~

3.5. To apply stimulation, set the microscope to scan for 15 to 20 cycles **[1]**. After completing cycles 1 to 5 to generate a baseline **[2]**, apply stimulation during scans 6 to 10. Wait for at least 5 minutes after each stimulus to prevent neuronal desensitization **[3]**. NOTE: The authors requested to skip these shots.

~~3.5.1. SCREEN: To be provided by authors: Setting the scan range to 15–20 cycles on the microscope interface.~~

~~3.5.2. SCREEN: To be provided by authors: A baseline is generated after completing cycles 1 to 5.~~

~~3.5.3. SCREEN: To be provided by authors: The stimulus being applied during cycle 6.~~

3.6. For von Frey stimulation targeting the V2 region of the trigeminal ganglion, hold the filament and apply it repeatedly to the ipsilateral area below the eye and above the mouth, from immediately after scan 5 until immediately after scan 10 **[1]**. For V3 region stimulation, apply the filament repeatedly to the ipsilateral area just below the ear during the same scanning window **[2]**.

3.6.1. Talent holding the filament and applying it to the ipsilateral area below the eye and above the mouth.

3.6.2. Talent holding the filament and applying it to the ipsilateral area just below the ear.

3.7. For cold and heat stimuli, cool or heat a beaker of water to just below or above the desired temperature **[1]**. Begin scanning, and immediately after scan 5 **[2]**, use a plastic transfer pipette to apply the thermal stimulus to the ipsilateral area below the eye and above the mouth for the V2 region **[3]**, or just below the ear for the V3 region **[4]**.

3.7.1. Talent heating or cooling the beaker and adjusting the water temperature in the beaker using a thermometer.

3.7.2. Talent starting the scan. *Videographer: Record the screen for this shot.*

3.7.3. Talent using a plastic transfer pipette to apply the thermal stimulus to the ipsilateral area below the eye and above the mouth.

3.7.4. Talent using a plastic transfer pipette to apply the thermal stimulus to the ipsilateral area below the ear.

3.8. At the end of the experiment, inject 50 millimolar potassium chloride subcutaneously into the V2 or V3 regions starting on cycle 6 to activate and identify all responsive neurons **[1]**. For the calcium imaging, calculate the calcium transient intensity using the given equation **[2]**. NOTE: The VO has been edited.

3.8.1. Talent injecting potassium chloride subcutaneously into either V2 or V3 area.

4.4.1. TEXT on PLAIN BACKGROUND:

$\Delta F/F_0 = (F_t - F_0)/F_0$

F_t: The pixel intensity in the ROI at the time point of interest

F_0: The baseline intensity

The baseline intensity: The mean intensity of the 2 – 4 frames preceding the Ca^{2+} transient (for spontaneous activity)

Or,

The baseline intensity: The mean intensity of the first 1 – 5 frames for stimulus-induced Ca^{2+} transients

For analysis, randomly select equal numbers of neurons from each ganglion to avoid sampling bias

NOTE: This shot is moved here as the authors requested to remove all shots in section 4, but wanted to have 4.4.1.

4. Calcium Imaging Analysis

~~4.1. Open the orthogonal projection file by dragging and dropping it into the analysis software **[1].** Select the image type by clicking on **Image**, then **Type**, and choosing **RGB Color [2].** To open the ROI *(R-O-I)* Manager, click on **Analyze**, then **Tools**, and select **ROI Manager [3].**~~

~~4.1.1. SCREEN: To be provided by authors: Drag and drop the orthogonal projection file into the software.~~

~~4.1.2. SCREEN: To be provided by authors: Click on **Image**, select **Type**, and choose **RGB Color** from the dropdown menu.~~

~~4.1.3. SCREEN: To be provided by authors: Click on **Analyze**, go to **Tools**, and open the **ROI Manager** window.~~

4.2. Select active neurons by drawing an ROI using the **Ellipse** tool in the toolbar **[1]**. Add each selected region to the ROI file by clicking the **Add** button in the ROI Manager window **[2]**.

4.2.1. SCREEN: To be provided by authors: Using the **Ellipse** tool to draw around an active neuron.

4.2.2. SCREEN: To be provided by authors: Adding the selected region to the ROI file by clicking the **Add** button in the ROI Manager.

4.3. Now, go to **Analyze**, select **Measurement Settings**, and check the option for **Mean Gray Value [1]**. In the ROI Manager window, click **More** and then choose **Multi-Measure** to measure the intensity **[2]**. When a new window labeled "Results" opens, save the CSV file by selecting **File**, then **Save As [3]**. Open the CSV file in a spreadsheet software and save it as a spreadsheet file **[4]**.

4.3.1. SCREEN: To be provided by authors: Click on **Analyze**, select **Measurement Settings**, and check **Mean Gray Value**.

4.3.2. SCREEN: To be provided by authors: In the ROI Manager, open the **More** menu and select **Multi-Measure** to measure the intensity.

4.3.3. SCREEN: To be provided by authors: Display of the "Results" window with the generated CSV file, and clicking on **File**, then **Save As** to save the CSV file.

4.3.4. SCREEN: To be provided by authors: Opening the saved CSV file in a spreadsheet software and saving it as a spreadsheet file.

4.4. Calculate the calcium transient intensity using the given equation **[1]**. For the analysis, randomly sample approximately the same number of neurons from each ganglion to ensure that one ganglion does not dominate the analysis **[2]**.

4.4.1. TEXT on PLAIN BACKGROUND:

$\Delta F/F_0 = (F_t - F_0)/F_0$

F_t: The pixel intensity in the ROI at the time point of interest

F_0: The baseline intensity

The baseline intensity: The mean intensity of the 2 – 4 frames preceding the Ca^{2+} transient (for spontaneous activity)

Or,

The baseline intensity: The mean intensity of the first 1 – 5 frames for stimulus-induced Ca^{2+} transients

~~For analysis, randomly select equal numbers of neurons from each ganglion to avoid sampling bias~~ NOTE: 4.4.1 is moved below 3.8.1.

~~4.4.2. SCREEN: To be provided by authors: Randomly sampling approximately the same number of neurons from the ganglion.~~

~~4.5. Measure neuron diameter by using the Line tool in the toolbar to draw lines along the longest and shortest diameters of each neuron **[1]**, then calculate the average of those measurements **[2]**.~~

~~4.5.1. SCREEN: To be provided by authors: Using the Line tool to draw and measure the longest and shortest diameters of a neuron.~~

~~4.5.2. SCREEN: To be provided by authors: Calculating the average of the measurements.~~

Results

5. Results

5.1. Confocal imaging of the trigeminal ganglion enabled simultaneous visualization of over 3,000 neurons, capturing both spontaneous and stimulus-evoked calcium transients across V2 and V3 regions **[1]**.

5.1.1. LAB MEDIA: Figure 4A.

5.2. In the absence of stimulation, a subset of these neurons exhibited spontaneous calcium transients with distinct activity profiles across six individually tracked cells **[1]**.

5.2.1. LAB MEDIA: Figure 4B, 4C. *Video Editor: Highlight the yellow arrowheads in B.*

5.3. Application of noxious heat to the maxillary region resulted in visible activation of multiple neurons as indicated by bright fluorescence **[1]**, with synchronized increases in calcium transient intensity **[2]**.

5.3.1. LAB MEDIA: Figure 4E, 4F. *Video Editor: Highlight the yellow arrowheads in E.*

5.3.2. LAB MEDIA: Figure 4E, 4F. *Video Editor: Emphasize 4F.*

5.4. Noxious heat applied to the mandibular region similarly activated distinct neurons **[1]**, with variable patterns of calcium responses across individual cells **[2]**.

5.4.1. LAB MEDIA: Figure 4H, 4I. *Video Editor: Highlight the yellow arrowheads in H.*

5.4.2. LAB MEDIA: Figure 4H, 4I. *Video Editor: Emphasize 4I.*

5.5. A significantly higher number of neurons responded to 2-gram von Frey stimulation **[1]** compared to 0.4 grams in the V2 region **[2]**.

5.5.1. LAB MEDIA: Figure 5A. *Video Editor: Highlight the 2 gm bar.*

5.5.2. LAB MEDIA: Figure 5A. *Video Editor: Highlight the 0.4 gm bar.*

5.6. The calcium transient intensity in individual neurons increased more strongly with 2 gram stimulation than with 0.4 gram, with a higher peak and broader response spread **[1]**.

5.6.1. LAB MEDIA: Figure 5B. *Video Editor: Highlight the bottom graph (2 gm).*

5.7. Mean calcium response intensity during the first stimulus frame was also significantly greater following 2-gram stimulation compared to 0.4-gram **[1]**.

5.7.1. LAB MEDIA: Figure 5C. *Video Editor: Highlight the 2.0 g plot (top one).*