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Title: Cell Subtype-Specific Analysis of Neuronal Membrane Proteasome in Somatosensory Neurons

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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: 27
Number of Shots: 50

Introduction

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

1.1. **Eric Villalón:** We are investigating the mechanisms of how somatosensory neurons communicate through the recently discovered neuronal membrane proteasome (NMP) to modulate how we sense touch, itch, and pain.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

What significant findings have you established in your field?

1.2. **Meghan Imhoff:** We recently discovered the neuronal membrane proteasome, a specialized protein degradation complex found in some sensory neurons, which function to modulate sensitivity to touch, itch, and pain.

1.2.1. 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?

1.3. **Eric Villalon:** With this protocol, we will be able to investigate the expression and modulation dynamics of the NMP in a cell-type-specific manner in health and disease conditions.

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

What advantage does your protocol offer compared to other techniques?

1.4. **Meghan Imhoff :** Our protocol enables the isolation of NMP-expressing neurons from the non-NMP-expressing neurons to be able to study their function with cell-type specificity. Importantly, these isolated neuronal populations remain viable for downstream analyses. *Suggested B-roll: Figure 2*

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

What questions will future research focus on?

1.5. **Meghan Imhoff:** With these protocols, we will begin to investigate how different neuropathic conditions affect the expression and function of the NMP and how this contributes to altered touch, itch, and pain sensation.

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 Loyola University of Chicago Stritch School of Medicine

Protocol

2. Preparation of Single-Cell Dorsal Root Ganglia (DRG) Neuron Suspension

Demonstrator: Meghan Imhoff

2.1. To begin, transfer the harvested dorsal root ganglia into a 15-milliliter conical tube **[1-TXT]**. Using a pipette, add 7 milliliters of tissue dissociation enzyme blend working solution to the tube **[2]** and incubate at 37 degrees Celsius with gentle agitation for 20 minutes **[3]**.

2.1.1. WIDE: Talent transferring dissected dorsal root ganglia into a labeled 15 milliliter conical tube. **TXT: Harvest DRGs from 4–6 P20–P23 mice**

2.1.2. Talent pipetting 7 milliliters of enzyme solution into the conical tube.

2.1.3. Talent placing the tube on a rotating incubator set at 37 degrees Celsius.

2.2. Place the tube in a centrifuge and spin down the ganglia at 120 *g* for 2 minutes **[1]**. Carefully aspirate the supernatant without disturbing the pellet **[2]**. Resuspend the ganglia in 7 milliliters of low trituration tissue dissociation enzyme blend with papain **[3-TXT]**.

2.2.1. Talent placing the conical tube in a centrifuge and starting the spin.

2.2.2. Talent aspirating the supernatant gently, avoiding the pellet.

2.2.3. Talent pipetting 7 milliliters of papain-containing enzyme solution into the tube and mixing it. **TXT: Incubate at 37 °C for 15 min**

2.3. After centrifuging the tube again, aspirate and discard the supernatant **[1]**, then resuspend the ganglia in 500 microliters of BSA *(B-S-A),* TI *(T-I)* and DMEM *(D-M-E-M)* solution **[3]**.

2.3.1. Talent aspirating the supernatant from the pellet.

2.3.2. Talent resuspending the pellet with 500 microliters of BSA/TI/DMEM using a pipette.

2.4. Using a 1 milliliter plugged fire-polished glass Pasteur pipette, triturate the ganglia 12 to 16 times **[1]**.

2.4.1. Talent triturating the ganglia by pipetting up and down using a plugged fire-polished Pasteur pipette.

2.5. Pass the cell suspension through a 40-micrometer cell strainer to filter out cellular debris **[1]**. Wash the strainer with 1 milliliter of BSA, TI, and DMEM solution to collect the remaining dorsal root ganglia neurons **[2]**. Transfer the filtered cell suspension into a 15-milliliter conical tube **[3-TXT]**.

2.5.1. Talent filtering the suspension through a 40 micrometer strainer positioned over a 3.5 cm culture plate.

2.5.2. Talent pipetting 1 milliliter of BSA/TI/DMEM solution to wash the strainer.

2.5.3. Talent transferring the filtered suspension into a clean 15 milliliter conical tube. **TXT: Centrifuge and resuspend the pellet in 1 ml DRG medium**

3. Cell-Type-Specific NMP Expression Analysis

3.1. Prepare an immunofluorescence staining tray by lining it with a large piece of parafilm **[1]**.

3.1.1. WIDE: Talent cutting a sheet of parafilm and placing it onto an immunofluorescence staining tray.

3.2. Using sterile 5-dash-45 forceps, transfer the coverslips from the tissue culture dish onto the parafilm, ensuring that the side containing dorsal root ganglia neurons faces upward **[1]**. Slowly pipette 100 microliters of dorsal root ganglia media onto each coverslip to prevent the cells from drying out **[2]**.

3.2.1. Talent using sterile angled forceps to gently lift each coverslip and place it neuron-side up on the parafilm-lined tray.

3.2.2. Talent pipetting 100 microliters of media just over the edge of each coverslip.

3.3. Then, dilute the goat anti-PSMA2 *(P-S-M-alpha-Two)* primary antibody in warm dorsal root ganglia media to achieve a 1 to 20 dilution **[1]**.

3.3.1. Talent adding goat anti-PSMA2 antibody into a tube with DRG medium.

3.4. Using an aspirator fitted with a P10 *(P-ten)* unfiltered tip, slowly remove the media from the edge of each coverslip **[1]**. Add 100 microliters of the goat anti-PSMA2 antibody solution to each coverslip and incubate at room temperature for 40 minutes **[2]**.

3.4.1. Talent aspirating the media slowly from the edge of the coverslip using an aspirator with a P10 unfiltered tip.

3.4.2. Talent adding 100 microliters of diluted primary antibody solution to each coverslip and keeping it aside.

3.5. After that, carefully aspirate the primary antibody solution from each coverslip **[1]**. Add 100 microliters of room-temperature PBS to the cells and incubate at room temperature for 3 minutes to wash **[2-TXT]**.

3.5.1. Talent aspirating the antibody solution from the edge of each coverslip.

3.5.2. Talent pipetting 100 microliters of room-temperature PBS onto each coverslip and keeping it aside. **TXT: Wash 3x with PBS**

3.6. Now, to prepare the secondary anti-goat antibody, dilute it in warm dorsal root ganglia media at a 1 to 250 dilution **[1]**.

3.6.1. Talent pipetting warm dorsal root ganglia media in a tube and adding 555-conjugated anti-goat secondary antibody.

3.7. After completing the third wash, add 100 microliters of the diluted secondary antibody solution to each coverslip **[1]**. Incubate the coverslips at room temperature for 40 minutes while protecting the cells from light **[2]**.

3.7.1. Talent pipetting 100 microliters of diluted secondary antibody onto each coverslip.

3.7.2. Talent placing a cover over the staining tray and keeping it aside.

3.8. Then, aspirate the secondary antibody solution from the coverslips **[1]** and wash the cells three times using PBS as described previously **[2]**.

3.8.1. Talent aspirating the secondary antibody from the edge of each coverslip.
Videographer's Note: Use second take. The authors said we can reuse another one because they're the same.

3.8.2. Talent adding PBS on the coverslip and removing it. *Videographer: Please film multiple reusable takes to use them later*
Videographer's Note: Will be in two parts because there's an incubation period after adding PBS on the coverslip.

3.9. After the final PBS wash, add 100 microliters of fixative solution containing 4 percent paraformaldehyde and 4 percent sucrose in PBS **[1]**.

3.9.1. Talent adding 100 microliters of fixative solution to each coverslip.

3.10. Once the cells are incubated at room temperature for 10 minutes, aspirate the fixative from each coverslip **[1]**, then wash the cells three times with PBS as previously

described **[2]**.

3.10.1. Talent aspirating the fixative solution from the coverslips.

3.10.2. *Reuse 3.8.2*

3.11. To permeabilize the cells, add 100 microliters of 0.1 percent nonionic detergent in PBS. Incubate the coverslips at room temperature for 5 minutes **[1]**.

3.11.1. Talent pipetting 100 microliters of 0.1 percent Triton X-100 solution onto each coverslip and keeping it aside.

3.12. Then, aspirate the permeabilizing solution from the coverslips **[1]** and wash the cells three times using PBS as previously described **[2]**.

3.12.1. Talent aspirating the Triton X-100 solution from the coverslips.

3.12.2. *Reuse 3.8.2*

3.13. Add 100 microliters of blocking solution composed of 5 percent fetal bovine serum and 5 percent donkey serum in PBS to each coverslip **[1-TXT].**

3.13.1. Talent pipetting 100 microliters of blocking solution onto each coverslip. **TXT: Incubate at RT for 40 min**

3.14. Next, dilute rabbit anti-CGRP *(C-G-R-P)*, isolectin B4 biotin conjugate, and chicken anti-NF-H *(N-F-H)* in the blocking solution to prepare the dorsal root ganglion neuron cell-type-specific primary antibody mixture **[1]**.

3.14.1. Talent sequentially adding rabbit anti-CGRP, isolectin B4 biotin conjugate, and chicken anti-NF-H antibodies to labelled tubes.

3.15. Aspirate the blocking solution from the coverslips **[1]**, then add 100 microliters of the prepared primary antibody mixture to each coverslip **[2]**. Incubate the coverslips at room temperature for 45 minutes while protecting them from light **[3]**.

3.15.1. Talent aspirating the blocking solution from each coverslip.

3.15.2. Talent adding 100 microliters of mixed primary antibody solution to each coverslip.

3.15.3. Talent covering the tray to protect samples during the 45-minute incubation.

3.16. Afterward, remove the primary antibody solution from the coverslips **[1]**, and wash the cells three times using PBS as previously described **[2]**.

3.16.1. Talent aspirating the antibody mixture from the edge of each coverslip.

3.16.2. *Reuse 3.8.2*

3.17. Now, prepare the secondary antibody mixture by diluting streptavidin, donkey anti-rabbit, and donkey anti-chicken to 1 to 500 each in blocking solution **[1]**.

3.17.1. Talent pipetting blocking solution into a tube and adding antibody mix.

3.18. Aspirate the final PBS wash from each coverslip **[1]**, then add 100 microliters of the prepared secondary antibody solution **[2]**. Incubate the coverslips at room temperature for 45 minutes in the dark **[3-TXT]**.

3.18.1. Talent aspirating the last wash from the coverslips using a pipette.

3.18.2. Talent adding 100 microliters of secondary antibody mix to each coverslip.

3.18.3. Talent placing the tray ~~with foil and keeping it~~ aside. **TXT: Wash 3x with PBS**

3.19. Aspirate the final wash from the coverslip **[1]**. Using fine-tip forceps, lift each coverslip from the parafilm and gently blot off excess liquid by touching the edge of the coverslip to a lint-free tissue **[2]**.

3.19.1. Talent aspirating the last drop of water from each coverslip.

3.19.2. Talent lifting each coverslip with forceps and gently blotting the edge on a lint-free tissue.
Videographer's Note: 3.19.2 - Goes after 3.20.1. Combined with 3.20.2

3.20. Place a 7-microliter droplet of mounting medium on a microscope slide **[1]**. Carefully lower the coverslip onto the mounting medium with the cell side facing down, ensuring full contact with the medium **[2]**.

3.20.1. Talent pipetting 7 microliters of mounting medium onto the center of a clean microscope slide.

3.20.2. Talent positioning the coverslip over the droplet and gently placing it so the cell side faces down into the medium.

3.21. Place the prepared slides in a dark, dry drawer or box to dry for at least 1 hour **[1]**. Seal the edge of the coverslip with fast-dry nail polish and wait 10 to 15 minutes before imaging **[2-TXT]**.

3.21.1. Talent transfers the slides into a dark storage container or drawer for drying.

3.21.2. Talent sealing the edge of each coverslip with a layer of fast-dry nail polish and

waiting. **TXT: Image using a fluorescent or spinning-disk confocal microscope**

Results

4. Results

4.1. Antibody feeding on live dorsal root ganglion neurons revealed a subpopulation of neurons with surface-accessible proteasomes **[1],** while control samples lacking primary antibody showed no surface labeling **[2].**

4.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the red-stained cells labeled "NMP$^+$" indicated by red arrows.*

4.1.2. LAB MEDIA: Figure 2B.

4.2. Flow cytometry identified two distinct populations of dorsal root ganglion neurons based on fluorescence intensity, separating NMP *(N-M-P)*-positive from NMP-negative cells **[1],** with clear gating regions established using controls **[2].**

4.2.1. LAB MEDIA: Figure 3A (right panel). *Video editor: Highlight the regions shown using boxes*

4.2.2. LAB MEDIA: Figure 3A (left panel). *Video editor: show absence of cells in the boxed regions*

4.3. Quantification of flow-sorted populations showed that approximately 4% of dorsal root ganglion neurons were NMP positive, while the remaining majority were NMP negative **[1].**

4.3.1. LAB MEDIA: Figure 3B.

4.4. Sorted NMP positive and NMP negative neurons retained viability and expressed cell-type-specific markers NF-H *(N-F-H)* **[1],** IB4 *(I-B-Four)* **[2],** and CGRP *(C-G-R-P)*, confirming the suitability of the workflow for downstream molecular analysis **[3].**

4.4.1. LAB MEDIA: Figure 4. *Video editor: Highlight the left panel.*

4.4.2. LAB MEDIA: Figure 4 *Video editor: Highlight the middle panel.*

4.4.3. LAB MEDIA: Figure 4 *Video editor: Highlight the right panel.*

Pronunciation Guide:

- **Proteasome**
  Pronunciation link: https://www.merriam-webster.com/medical/proteasome Merriam-Webster
  (Also see audio examples at HowToPronounce) How To Pronounce
  IPA: /ˈproʊ.ti.ə.soʊm/
  Phonetic spelling: PROH-tee-uh-sohm

- **Somatosensory**
  Pronunciation link: https://www.merriam-webster.com/dictionary/somatosensory Merriam-Webster
  (Also see Forvo) Forvo.com
  IPA: /ˌsoʊ.mə.təˈsɛn.səˌri/
  Phonetic spelling: SOH-muh-tuh-SEN-suh-ree

- **Dorsal**
  Pronunciation link: https://www.merriam-webster.com/dictionary/dorsal (you can check there)
  IPA: /ˈdɔr.səl/
  Phonetic spelling: DOR-suhl

- **Ganglia**
  Pronunciation link: https://www.merriam-webster.com/dictionary/ganglia
  IPA: /ˈgæŋ.li.ə/
  Phonetic spelling: GANG-lee-uh

- **Immunofluorescence**
  Pronunciation link: (no single Merriam-Webster page for the full word, but "immuno-" and "fluorescence" separately)
  IPA: /ɪˌmju.noʊ.flʊˈrɛs.əns/
  Phonetic spelling: im-yoo-noh-floo-RESH-əns

- **Permeabilize**
  Pronunciation link: https://www.merriam-webster.com/dictionary/permeabilize
  IPA: /pərˈmiː.ə.bə.laɪz/
  Phonetic spelling: per-MEE-uh-by-lyze

- **Isolectin**
  Pronunciation link: (no central dictionary entry for "isolectin")
  IPA (approximate): /ˌaɪ.soʊˈlɛk.tɪn/
  Phonetic spelling: EYE-soh-LEK-tin

- **Streptavidin**
  Pronunciation link: (no common dictionary entry, but standard in biochemistry)
  IPA (approximate): /streptəˈvɪdɪn/
  Phonetic spelling: strep-tuh-VID-in

- **Paraformaldehyde**
  Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
  IPA: /ˌpær.ə.fɔːrˈmæl.də.haɪd/
  Phonetic spelling: par-uh-for-MAL-duh-hyde