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Title: Examination of Anatomical Features of Retinal Ganglion Cells under N-Methyl-D-Aspartic Acid (NMDA)-Induced Excitotoxicity

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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

   If **Yes**, can you record movies/images using your own microscope camera?

   NO

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

   If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

   Zeiss Stemi 508

   SCOPE shots: 2.3.1, 2.3.2, 2.3.3, 2.3.4, 2.3.5, 2.3.6, 2.4.1, 2.4.2, 2.4.3, 2.5.1, 2.5.2, 2.5.3, 2.5.4, 2.6.1, 2.6.2, 2.6.3, 2.7.1, 2.7.2

Videographer: Please film the above-mentioned shots using the scope kit

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? **Yes**

If **Yes**, how far apart are the locations? 1 min walking distance

Current Protocol Length
Number of Steps: 20
Number of Shots: 48 (18 Scope)

Introduction

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

1.1. **Chai-An Mao:** We are trying to understand development, survival and degeneration of retinal ganglion cells using murine models.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What technologies are currently used to advance research in your field?

1.2. **Chai-An Mao:** We are using genetically modified mouse lines to label specific cell types to monitor the degeneration of these cells.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

What advantage does your protocol offer compared to other techniques?

1.3. **Ashlyn Tu:** With this protocol, we can investigate anatomical futures and phenotypes of retinal ganglion cells under NMDA-induced excitotoxicity.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.8.1*

How will your findings advance research in your field?

1.4. **Ashlyn Tu:** This approach provides new insights into how distinct retinal cells respond to NMDA at gross and subcellular levels.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.11.1*

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at The University of Texas McGovern Medical School at Houston

Protocol

2. Tissue Collection and Retinal Processing for Staining

Demonstrator: Ashlyn Tu

2.1. To begin, obtain the eyeball from the NMDA-injected mouse after euthanization **[1-TXT]** and transfer it into a separate labeled 2 milliliter tube **[2]**.

2.1.1. WIDE: Talent picking up the eyeball from a container using forceps. **TXT: NMDA: N-Methyl-D-Aspartic Acid** Authors, please **do not** show the animal or the dissection procedure

2.1.2. Talent placing the eyeball into a labeled 2.0 milliliter tube.

2.2. Fix the eyeball in 10 percent neutral buffered formalin for 10 minutes at room temperature **[1]** and obtain a cellulose nitrate membrane filter to flat mount the retina **[2]**.

2.2.1. Talent adding 10 percent formalin to the eyeball.

2.2.2. Talent placing a cellulose nitrate membrane filter beside the dissection board.

2.3. After fixation, transfer the eyeball to a 10-centimeter Petri dish containing 1X PBS **[1]**. Using forceps, hold any muscle tissue attached to the eyeball **[2]** and create a hole at the limbus with a 30-gauge injection needle **[3]**. Insert the tip of Vanna scissors into the hole **[4]** and cut around the ora serrata **[5]**. Using forceps, remove the lens from the eyecup **[6]**.

2.3.1. SCOPE: transferring eyeballs into a Petri dish filled with 1X PBS.

2.3.2. SCOPE: using forceps to hold the muscle tissue on the eyeball.

2.3.3. SCOPE: the limbus being punctured with a 30 gauge needle.

2.3.4. SCOPE: inserting Vanna scissors into the hole.

2.3.5. SCOPE: cutting around the ora serrata.

2.3.6. SCOPE: removing the lens with forceps.

Videographer: Please film the SCOPE shots using the scope kit

2.4. Then, make 3 to 4 cuts on the eyecup **[1]**. Use forceps to hold the retinal pigment epithelium **[2]** and gently peel it off from the retina **[3]**.

2.4.1. SCOPE: making multiple cuts on the eyecup.

2.4.2. SCOPE: delicately holding the retinal pigment epithelium.

2.4.3. SCOPE: peeling off the retinal pigment epithelium using forceps.

2.5. Now, cut a small piece of cellulose nitrate membrane filter **[1-TXT]** and soak it in 1X PBS **[2]**. Place the isolated retina onto the soaked filter **[3]** and use a fine paintbrush to gently push a peripheral area of the retina to attach its edge to the filter **[4]**.

2.5.1. SCOPE: cutting the membrane filter. **TXT: Trim the filters into different shapes to distinguish between samples**

2.5.2. SCOPE: dunking the piece of membrane filter in PBS.

2.5.3. SCOPE: placing the retina onto the soaked filter.

2.5.4. SCOPE: using a fine paintbrush to attach the edge of the retina to the filter.

2.6. Next, hold the edge of the filter and slowly pull it out of the PBS **[1]**, ensuring the retina stays attached and flattens onto the filter **[2]**. Place the filter on a paper towel for 1 minute to secure the attachment **[3]**.

2.6.1. SCOPE: pulling the filter with the retina out of the PBS.

2.6.2. SCOPE: Shot of retina attached and flattened on the filter.

2.6.3. SCOPE: placing the filter on a paper towel for one minute.

2.7. Then, transfer the retina and the filter back to a Petri dish containing 1X PBS **[1]**. Using forceps and a fine paintbrush, carefully remove any debris, hair, and vitreous from the retina as much as possible **[2]**.

2.7.1. SCOPE: Talent placing the retina/filter back into a Petri dish.

2.7.2. SCOPE: Talent cleaning the retina with forceps and a fine paintbrush.

2.8. Transfer the retina and filter back into a Petri dish **[1]** and incubate the retina with 10 percent neutral buffered formalin for 5 minutes at room temperature with gentle agitation **[2]**.

2.8.1. Talent transferring the retina/filter back into a Petri dish.

2.8.2. Talent placing the dish with retina and formalin on a slow rocker.

2.9. Now, prepare a 65-degree Celsius water bath **[1]** to preheat 30 milliliters of 1X PBS in a 50-milliliter glass beaker **[2]**. Transfer the retina and filter directly into the preheated PBS **[3]** and incubate it for 30 minutes **[4]**.

2.9.1. Talent switching on a 65 degree Celsius water bath.

2.9.2. Talent placing the beaker with 30 milliliters of PBS in the heated bath.

2.9.3. Talent placing retina/filter into the warm PBS.

2.9.4. Close-up of the retina dunked in the PBS.

3. Alkaline Phosphatase Staining and Imaging of the Retina

3.1. After heat treatment, transfer the retina into a 30-millimeter Petri dish containing alkaline phosphatase or AP buffer **[1-TXT]**.

3.1.1. Talent placing retina into a 30 millimeter Petri dish filled with alkaline phosphatase buffer. **TXT: Buffer contains: 100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$**

3.2. Incubate the retina in the alkaline phosphatase buffer for 5 minutes at room temperature with gentle agitation **[1]**.

3.2.1. Talent placing the dish with retina and AP buffer on a slow rocker at RT.

3.3. After incubation, replace the alkaline phosphatase buffer with the staining solution **[1-TXT]**.

3.3.1. Talent aspirating AP buffer and pouring staining solution into the Petri dish. **TXT: Staining solution: 30 mL of 10 mg/mL BCIP + 60 mL of 10 mg/mL NBP in 10 mL of AP buffer**

3.4. Then, place the Petri dish in a light-excluding box **[1]**. Develop the AP reaction at room temperature for 1 to 2 days with gentle agitation, until the staining intensity is suitable for imaging **[2]**.

3.4.1. Talent placing the dish inside a dark box.

3.4.2. Talent placing the box with retina-staining solution on a rocker.

3.5. To stop the reaction, wash the retina twice with 1X PBS for 5 minutes each at room temperature with gentle agitation **[1]**.

3.5.1. Talent aspirating the stain and adding PBS to the retina.

3.6. Then, postfix the retina in 10 percent neutral buffered formalin for 10 minutes at room temperature with gentle agitation **[1-TXT]**.

3.6.1. Talent adding formalin to the Petri dish and placing the dish on a gentle rocker.
TXT: Wash the retina 3x with PBS; 5 min each; RT; Gentle agitation

3.7. After washing the retina, dehydrate it in 30 percent ethanol followed by 50 percent ethanol for 30 minutes each at room temperature with gentle agitation **[2]**.

3.7.1. Talent placing the retina in a dish/jar containing ethanol.

3.8. Then, using a fine paintbrush, gently remove the retina from the filter paper **[1]** and dehydrate the filter-free retina in 70% ethanol, followed by 100% ethanol **[2]**.

3.8.1. Talent lifting retina off the filter using a paintbrush.

3.8.2. TEXT ON PLAIN BACKGROUND:

70% Ethanol (30 min)

100% Ethanol (2x, 30 min each)

RT

Gentle agitation

3.9. Post-dehydration, add benzyl benzoate and benzyl alcohol solution mixed in a 2 to 1 volume ratio to the retina **[1]** and incubate for 30 minutes to 2 hours at room temperature with gentle agitation **[2]**.

3.9.1. Talent adding the clearing solution to the retina.

3.9.2. Talent placing the dish on a gentle rocker.

3.10. To mount the retina, apply double-sided tape to a glass slide to create a platform for the coverslip and retina **[1]** and place the retina on the slide **[2].** Fill the space with clearing solution **[3]**, apply the coverslip **[4]**, and seal the slide with nail polish to secure the retina and coverslip **[5]**.

3.10.1. Talent sticking double-sided tape onto the slide to create a raised area.

3.10.2. Talent placing retina in position on the slide.

3.10.3. Talent filling the space with clearing solution.

3.10.4. Talent placing the coverslip on the sample.

3.10.5. Talent applying nail polish to seal the sample.

3.11. Finally, image the AP-stained retina using an apotome microscope **[1]**.

3.11.1. Talent placing the sample under the Apotome microscope.

Results

4. Results

4.1. The NMDA injection reduced the number of RNA-binding proteins with multiple splicing-positive retinal ganglion cells by approximately 65% **[1]** compared to controls, as revealed by immunofluorescence staining one week post-injection **[2]**.

4.1.1. LAB MEDIA: Figure 2EFG *Video editor: Highlight the panel F and the NMDA bar in G*

4.1.2. LAB MEDIA: Figure 2EFG *Video editor: Highlight the panel E and the Control bar in G*.

4.2. The number of Tbr2 *(T-B-R-2)*-expressing retinal ganglion cells and amacrine cells was drastically reduced 1 week after NMDA injection, indicating that Tbr2-expressing cells are not entirely resistant to NMDA-induced damage **[1]**.

4.2.1. LAB MEDIA: Figure 2 A B. *Video editor: Highlight B*.

4.3. Pcp2-expressing on-off retinal ganglion cells exhibited a visible reduction in number and staining intensity in NMDA-injected retinas **[1]** compared to the control **[2]**.

4.3.1. LAB MEDIA: Figure 2 C D. *Video editor: Highlight D*

4.3.2. LAB MEDIA: Figure 2 C D. *Video editor: Highlight C*

4.4. Tbr2-expressing retinal ganglion cells and amacrine cells under NMDA insult showed dendritic arbor shrinkage, collapse, and aggregation, with fragmented dendrites frequently appearing near degenerating cells **[1]**.

4.4.1. LAB MEDIA: Figure 3 ABC. *Video editor: Zoom in at the dark fragments aggregating at centres in images A B C*

4.5. Tbr1-expressing off retinal ganglion cells exhibited missing soma and proximal dendrites, along with fragmented distal dendrites, 1 week after NMDA exposure **[1]**.

4.5.1. LAB MEDIA: Figure 4 A B. *Video editor: Highlight the dotted circles in panel B*

4.6. Immunofluorescence staining of Tbr1-expressing J-type retinal ganglion cells revealed substantial loss of dendritic arbor under NMDA insult **[1]**.

4.6.1. LAB MEDIA: Figure 4 C D. *Video editor: Highlight circled region in panel D* .

Pronunciation guides

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1. euthanization

- Pronunciation link: https://www.howtopronounce.com/euthanization (howtopronounce.com)
- **IPA:** /juːˌθænəˈzeɪʃən/
- **Phonetic spelling:** yoo-tha-nuh-ZAY-shuhn

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2. formalin

- Pronunciation link: No confirmed link found
- **IPA:** /ˈfɔːrməlɪn/
- **Phonetic spelling:** FOR-muh-lin

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3. cellulose

- Pronunciation link: No confirmed link found
- **IPA:** /ˈsɛljəˌloʊs/
- **Phonetic spelling:** SEL-yuh-lohs

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4. retina

- Pronunciation link: No confirmed link found
- **IPA:** /ˈrɛtɪnə/
- **Phonetic spelling:** RET-i-nuh

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5. limbus

- Pronunciation link: No confirmed link found
- **IPA:** /ˈlɪmbəs/
- **Phonetic spelling:** LIM-bus

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6. ora serrata

- Pronunciation link: No confirmed link found
- **IPA:** /ˌɔːrə sɛˈrɑːtə/
- **Phonetic spelling:** OR-uh seh-RAH-tuh

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7. retinal pigment epithelium

- Pronunciation link: No confirmed link found
- **IPA:** /ˈrɛtɪnəl ˈpɪgmənt ˌɛpɪˈθiːliəm/
- **Phonetic spelling:** RET-i-nuhl PIG-muhnt ep-ih-THEE-lee-um

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8. benzyl benzoate

- Pronunciation link: No confirmed link found
- **IPA:** /ˈbɛnzaɪl ˈbɛnzoʊeɪt/
- **Phonetic spelling:** BEN-zyl BEN-zoh-ayt

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9. benzyl alcohol

- Pronunciation link: No confirmed link found
- **IPA:** /ˈbɛnzaɪl ˈælkəhɒl/
- **Phonetic spelling:** BEN-zyl AL-kuh-hol

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10. apotome

- Pronunciation link: No confirmed link found
- **IPA:** /əˈpɑːtoʊm/
- **Phonetic spelling:** uh-PAH-tohm

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11. immunofluorescence

- Pronunciation link: No confirmed link found
- **IPA:** /ɪˌmjuːnoʊˈflʊərəˌsɛns/

- **Phonetic spelling:** im-yoo-noh-FLOOR-uh-sens

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12. dendritic

- Pronunciation link: No confirmed link found
- **IPA:** /dɛnˈdrɪtɪk/
- **Phonetic spelling:** den-DRIT-ik

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13. amacrine

- Pronunciation link: No confirmed link found
- **IPA:** /ˈæməˌkraɪn/
- **Phonetic spelling:** AM-uh-krayn

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14. ganglion

- Pronunciation link: No confirmed link found
- **IPA:** /ˈgæŋgliən/
- **Phonetic spelling:** GANG-lee-un

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15. apoptosis

- Pronunciation link: https://www.merriam-webster.com/dictionary/apoptosis (merriam-webster.com)
- **IPA:** /ˌæpəpˈtoʊsɪs/
- **Phonetic spelling:** ap-uhp-TOH-sis
