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Title: Immunostaining of whole-mount retinas with the CLARITY tissue clearing method

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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?

   YES

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

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

Current Protocol Length

Number of Steps: 21
Number of Shots: 40

Introduction

1. Introductory Interview Statements

1.1. **Elizabeth Alessio:** This protocol allows for the investigation of the fine morphology of retinal neurons and can give insight into the cellular and sub-cellular morphological changes that occur in disease states.

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

1.2. **Elizabeth Alessio:** This method significantly improves the optical transparency of the retina and allow for high-resolution-3-dimensional imaging of circuit wiring and fine subcellular structures of retinal neurons in whole-mount retina preparation.

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

1.3. **Ethics Title Card**
Mouse sample handling was conducted according to the National Institutes of Health guidelines for laboratory animals and was approved by the Institutional Animal Care and Use Committees at Oakland University.

Protocol

2. Tissue preparation

2.1. Enucleate the mouse eyes with curved forceps and transfer them into a small Petri dish with 0.1 molar PBS **[1]**. Poke a small hole along the cornea-sclera junction with a needle under the dissection microscope **[2]**. Then, transfer the eye into 4% paraformaldehyde for 1 hour **[3]**.

2.1.1. Establishing shot of talent doing enucleation of mouse eyes and transferring into petri dish.

2.1.2. SCOPE: 2.1.2.avi. Talent poking a small hole along the cornea-sclera junction under dissection microscope. NOTE: All SCOPE shots are uploaded to AWS

2.1.3. SCOPE: 2.1.3.avi. Talent transferring eye into paraformaldehyde solution.

2.2. Transfer the eye back to a dish with PBS **[1]**. Under a dissection microscope, use dissection scissors to cut all the way around the cornea-sclera junction **[2]**. Remove the cornea and lens **[3]**, then cut at the base of the optic nerve and carefully peel the sclera off with forceps to isolate the retina **[4]**. *Videographer: This step is difficult and important!*

2.2.1. Talent transferring eye to the PBS.

2.2.2. SCOPE: 2.2.2.avi. Talent making a cut around cornea-sclera junction.

2.2.3. SCOPE: 2.2.3.avi. Talent putting cornea and lens apart.

2.2.4. SCOPE: 2.2.4.avi. Talent making a cut to separate retina.

2.3. Make four small cuts evenly around the retina **[1]** and use a fine tip brush dipped in PBS to lay it flat, GCL side down, in a clover-like shape on a small square cut from nitrocellulose filter paper **[2]**. *Videographer: This step is difficult and important!*

2.3.1. SCOPE: 2.3.1.avi. Talent making cuts around retina.

2.3.2. SCOPE: 2.3.2.avi. Talent positioning the retina on the nitrocellulose with the brush.

2.4. Pick up the corner of the nitrocellulose paper with forceps and place it in a 48-well plate with 4 percent paraformaldehyde for 1 hour **[1-TXT]**. Then, transfer the filter paper and retina to a well with PBS and wash three times for 5 minutes each **[2]**.

2.4.1. Talent transferring the retina into 48 well plate. **TXT: Do not touch mounted retina**

2.4.2. Talent transferring retina to a well and giving PBS washes.

2.5. Thaw A4P0 solution on ice **[0]**, then transfer the retina into the A4P0 solution and incubate overnight at 4 degrees Celsius with gentle agitation **[1]**.

2.5.0 Added shot: A4P0 solution thawing on ice.

2.5.1. Retina incubating with A4P0 solution.

2.6. Add vegetable oil into the well to completely cover the A4P0 solution **[1]**. Incubate in a water bath at 40 degrees Celsius for 3 hours with no shaking **[2],** then wash three times in PBS for 5 minutes per wash **[3-TXT]**.

2.6.1. Talent adding vegetable oil into the wells.

2.6.2. Talent placing the retina and filter paper in water bath for incubation. NOTE: Shot continuously with 2.6.1

2.6.3. Talent giving the washes and aspirating the oil. **TXT: Ensure all the oil has been rinsed off**

2.7. Incubate the retina in 10 percent sodium dodecyl sulphate at 40 degrees Celsius for two days with gentle shaking **[1-TXT]**. Then, transfer the filter paper and retina to PBS with Triton-X-100 and wash five times for 90 minutes per wash **[2]**.

2.7.1. Talent placing the retina and filter paper in water bath for incubation. **TXT: Replace SDS with fresh solution on the second day.**

2.7.2. Talent transferring the mixture into PBST.

2.8. After the final wash, store the retina at 4 degrees Celsius in PBST with 0.01 percent sodium azide or move directly to immunostaining **[1]**.

2.8.1. Talent putting the sample in the refrigerator.

3. Immunostaining and refractive index matching

3.1. Remove the retina from the filter paper by gently peeling it off with a fine tip brush in PBST **[1]**. Incubate it in primary antibody diluted in blocking solution for 2 days at 40 degrees Celsius with gentle shaking **[2-TXT]**. After the incubation, wash five times for 90 minutes in PBST **[3]**.

3.1.1. Talent removing retina from the filter paper.

3.1.2. Talent adding primary antibody and leaving the retina to incubate. **TXT: Refer to Table 1 and Table 2 of the manuscript** NOTE: Shot continuously with 3.1.1

3.1.3. Talent giving the washes.

3.2. Incubate the retina with appropriate secondary antibodies diluted in blocking solution for 2 days at 40 degrees Celsius with gentle shaking **[1-TXT]**. Protect the sample from light through the remainder of the procedure **[2-TXT]**. Wash the retina five times for 90 minutes in 0.02 molar phosphate buffer **[3]**.

3.2.1. Talent adding secondary antibody and leaving the retina to incubate. **TXT: Refer to Table 3 of the manuscript**

3.2.2. Talent protecting the mixture from light. TXT: Plate will be covered with a foil-covered box.

3.2.3. Talent washing the retina.

3.3. Finally, incubate the retina in sorbitol-based Refractive Index Matching Solution at 40 degrees Celsius overnight with gentle shaking **[1]**.
  3.3.1. Talent leaving the retina in the incubator.

4. Mounting

4.1. Outline a glass coverslip with a fine-tip permanent marker to mark a square boundary on the back of a glass microscope slide, leaving a small gap in one corner for excess mounting solution to escape. **[1-TXT]**.
  4.1.1. Talent doing the marking. **TXT: 18 mm by 18 mm by 1.5 mm**

4.2. Flip the slide over and use a syringe to trace the boundary with a thin line of silicone grease on the front of the slide **[1]**. Leave a small gap in one corner for excess mounting solution to escape **[2]**.
  4.2.1. Talent flipping the slide and using syringe to trace the boundary.
  4.2.2. Talent ensuring that small gap is there for excess mounting solution to escape. NOTE: Shot continuously with 4.2.1

4.3. Transfer the retina to the center of the bounded area and position it with a fine-tip brush so that it lies flat with the photoreceptor side against the glass slide **[1]**.
  4.3.1. Talent transferring the retina to glass slide.

4.4. Pipette approximately 60-microliters of sRIMS *(Pronounce 'ess-rims')* so that it covers the flattened retina and extends to one corner of the enclosure **[1]**. Ensure that the retina stays flat and in place **[2]**.
  4.4.1. Talent covering the retina with sRIMS solution.
  4.4.2. Talent ensuring that retina remains in place during addition. NOTE: Shot continuously with 4.4.1

4.5. Apply the coverslip starting from the corner with the sRIMS and slowly lower it until it touches the grease on all sides **[1-TXT]**. *Videographer: This step is important!*

  4.5.1. Talent applying the cover slip. **TXT: Avoid bubbles formation**

4.6. Place a stack of 3 cover slips on each side of the mounted retina as a spacer. Use the long edge of another slide to press down the coverslip so that the mount is flat and even **[1]**. Store the slides at 4 degrees Celsius until imaging **[2]**. *Videographer: This step is important!*

  4.6.1. Talent applying cover slips to the slide.
  4.6.2. Talent storing the slides.

5. Imaging

5.1. Begin by placing the slide on the microscope stage and locating the sample **[1]**. To obtain z-stacked images of samples, first focus on the signal in each channel individually and set the exposure time or scanning speed, for fluorescence or confocal microscopes, respectively **[2]**.
  5.1.1. Talent putting the slide on the microscope stage.
  5.1.2. Talent setting up the instrument.

5.2. Set the range for the z-stack either by manually setting the focal plane at the top and bottom of the desired range, or by setting the midpoint and then specifying a range around the midpoint **[1]**. Adjust the step size or number of slices as desired **[2]**.
  5.2.1. SCREEN: Talent setting up the instrument. *Videographer: Film the screen*
  5.2.2. SCREEN: Talent adjusting the step size. *Videographer: Film the screen* NOTE: Steps 5.2 and 5.3 were filmed continuously, please reference audio notes in the video

5.3. Capture the image and save the original file, then export it as a TIFF file or another desired format **[1]**.
  5.3.1. SCREEN: Talent acquiring the images and saving it in desired format. *Videographer: Film the screen*

5.4. Use image analysis software of choice to adjust the brightness and contrast in each channel until optimum clarity is achieved in both the single images and the 3-dimensional rendering of the z-stack **[1]**.
  5.4.1. SCREEN: Brightness and contrast adjusted on an image. *Videographer: Film the screen*

Results

6. Results: Improved 3D resolution of fine processes of retina by modified CLARITY tissue clearing method

6.1. When processed with the modified CLARITY protocol, complete optical transparency throughout the thickness of retina was observed **[1]** compared to non-processed control retinas **[2]**.
6.1.1. LAB MEDIA: Figure 1A. *Video Editor: Emphasize on arrows.*
6.1.2. LAB MEDIA: Figure 1B.

6.2. Z-stacked images for Arrestin labelled cones in ONL **[1]**, TH-labeled DACs in INL **[2]**, and RBPMS-marked RGCs in GCL are shown here **[3]**.
6.2.1. LAB MEDIA: Figure 2A. *Video Editor: Emphasize on arrows and arrowheads.*
6.2.2. LAB MEDIA: Figure 2B. *Video Editor: Emphasize on arrows and arrowheads.*
6.2.3. LAB MEDIA: Figure 2C. *Video Editor: Emphasize on arrows and arrowheads.*

6.3. The relative location of neurons throughout the entire thickness of retina was observed in the overlay image **[1]**.
6.3.1. LAB MEDIA: Figure 2D.

6.4. TH staining in CLARITY-processed whole-mount retinas **[1]** was compared with images obtained from standard preparation **[2]**. Dendrites and axon-like processes of DACs were more clearly revealed in a CLARITY processed retina **[3]** than in a standard retina **[4]**.
6.4.1. LAB MEDIA: Figure 3A, B. *Video Editor: Emphasize on top panel.*
6.4.2. LAB MEDIA: Figure 3C, D. *Video Editor: Emphasize on bottom panel.*
6.4.3. LAB MEDIA: Figure 3A.
6.4.4. LAB MEDIA: Figure 3C.

6.5. Axon-like processes of DACs exhibited complete ring-like structures in a CLARITY retina **[1]** compared to a standard retina **[2]**.
6.5.1. LAB MEDIA: Figure 3A. *Video Editor: Emphasize on insert part.*
6.5.2. LAB MEDIA: Figure 3C. *Video Editor: Emphasize on insert part.*

6.6. Ring-like structures of CLARITY retina taken using fluorescence microscopy **[1]** were almost identical to those observed using confocal microscopy **[2]**. The axon-like processes also ran toward the outer retina **[3]**.
6.6.1. LAB MEDIA: Figure 3B.
6.6.2. LAB MEDIA: Figure 3A.
6.6.3. LAB MEDIA: Figure 4A. *Video Editor: Emphasize on white arrows.*

6.7. Immunostaining against GluA2 and PSD-95 showed distinct puncta, revealing individual GluA2-containing AMPA receptors **[1]** and putative postsynaptic sites **[2]**, respectively. An overlay image showed some puncta on DAC processes **[3]**.
  6.7.1. LAB MEDIA: Figure 4B. *Video Editor: Emphasize on arrows.*
  6.7.2. LAB MEDIA: Figure 4C. *Video Editor: Emphasize on arrows.*
  6.7.3. LAB MEDIA: Figure 4D.

6.8. The points of putative colocalization were presented in X-Z **[1]**, Y-Z **[2]** and X-Y planes **[3]** and TH colocalized clearly with both GluA2 and PSD-95 **[4]**.
  6.8.1. LAB MEDIA: Figure 5 A1-A5. *Video Editor: Emphasize on upper row of the figure (A1-A5).*
  6.8.2. LAB MEDIA: Figure 5 B1-B5. *Video Editor: Emphasize on middle row of the figure (B1-B5).*
  6.8.3. LAB MEDIA: Figure 5 C1-C5. *Video Editor: Emphasize on lower row of the figure (C1-C5).*
  6.8.4. LAB MEDIA: Figure A4, B4 and C4.

Conclusion

7. Conclusion Interview Statements

7.1. **Elizabeth Alessio:** It is important that the retina remains flat during the hydrogel polymerization process to ensure that the cleared retina can lay flat for mounting and imaging.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3,.2.6, 4.3.*