

Immunohistochemistry on cryosections from embryonic and adult zebrafish eyes

Rosa A. Uribe^{1,2} and Jeffrey M. Gross^{1,2,3}

¹Section of Molecular Cell and Developmental Biology

²Institute for Cell and Molecular Biology

³Institute for Neuroscience

The University of Texas at Austin

Box C1000, 1 University Station

Austin, TX 78712

Corresponding Author:

Ph: (512) 471-1518

Fax: (512) 471-3878

jmgross@mail.utexas.edu

Introduction

This protocol describes methods for the fixation, cryosectioning and immunohistochemical detection of proteins in embryonic and adult zebrafish eyes. The methods described here can be easily adapted for use on other zebrafish tissues.

Materials

Reagents

DMSO [Dimethylsulfoxide may be harmful if absorbed through the skin or if its fumes inhaled. Wear appropriate gloves and safety glasses. Use in a chemical fume hood to prevent inhalation. Store in a tightly closed container, DMSO is combustible. Keep away from heat, sparks, and open flame]

Normal Goat Serum (NGS)

Paraformaldehyde, 4% *dissolved in 1X PBS*

Phosphate Buffered Solution (PBS), 1X

Primary Antibody

Secondary Antibody

Sucrose, 25% and 35% *both dissolved in 1X PBS*

Tween-20

Vectashield Mounting Medium (Vector Labs)

Equipment

Coverslips, No. 1 thickness

Cryostat

Cryomolds (Tissue Tek)

Eppendorf Tubes, 1.5 mL

Humid Chamber: *A Tupperware container with air-sealed lock and wet paper towels or kimwipes inside to provide moisture*

Microslides, 1.0 mm thick, pre-cleaned and gelatin-coated

PAP pen (Sigma)

Razor Blade

Slide jar or Coplin jar

Tissue Freezing Medium (Triangle Biomedical Sciences (TBS))

Methods

Fixation

1. Collect and fix whole zebrafish embryos or surgically removed adult eyes in 4% Paraformaldehyde (PFA) at 4°C overnight or at room temperature for 4-6 hours in eppendorf tubes.
 - a. *If collecting embryos before 48 hpf, make sure to remove the chorion with forceps prior fixation. If fixing adult eyes, euthanize fish and surgically remove eye with sharp needles. Make small incision in cornea to allow fix to enter eye.*
 - b. *Pause point: may leave specimen for 1-3 days at 4°C in PFA.*
2. Remove 4% PFA by using a pipette to gently remove solution. Rinse in 1x PBS 3 times, 5 minutes each for total time of 15 minutes.
3. Soak specimen in 25% Sucrose dissolved in 1x PBS at room temperature until embryos or eyes sink to the bottom of the tube (*time varies, but may take up to 2.5 hours*).
4. Remove 25% sucrose and add 35% sucrose dissolved in 1x PBS. Soak at room temperature until embryos or eyes sink to the bottom of the tube (again, time varies). *Pause point: may leave in 35% sucrose at 4 °C for up to a week.*

Cryosectioning

5. To line fish up in cryomolds:
 - a. Prepare desired amount of cryomolds by filling cryomolds with Tissue Freezing Medium (TFM) at room temperature. Take care not to get any bubbles in the molds.
 - b. Using one TFM-filled mold as a transfer dish, remove specimen(s) from Eppendorf tube and stir gently around in TFM-filled mold to wash out 35% Sucrose.
 - c. Transfer specimen(s) to a new TFM-filled cryomold. With a blunt needle, submerge embryos in the TFM and move embryos into a row that faces one side of the mold: line up fish head first such that their tails are facing center of mold and their heads are against the

- wall (**Figure 1**). Keep embryos as close to one another as possible and in a straight line. For adult eye, orient in mold with lens facing outward (**Figure 2**). If not interested in lens tissue, one may remove the lens during this step for easier cryosectioning.
- d. Carefully transfer to -80 °C to freeze. *Pause point: may store at -80°C indefinitely.*
6. To prepare for cryosectioning:
- Set Cryostat to -20 °C. Remove specimen block from cryomold inside the cryostat. Using a razor blade, carefully trim away all excess frozen medium around specimen.
 - Place TFM on center of chilled cryostage to which your sample will be placed. *Carefully place trimmed specimen onto this TFM with side to be sectioned facing up. Ensure that specimen block is as straight as possible. Add more TFM around periphery of sample on stage. Freeze for 1-2 minutes prior to sectioning.*
 - Transfer cryostage to cryostage holder.
7. Using the trim function on the cryostat set to between 30-60 microns, trim through block until a uniform section is made in the appropriate part of the sample. Quickly transfer section to room temperature gelatin-coated slide by allowing cryosection to gently melt onto slide. *Be careful not to section past the area of interest by checking sections using a basic light microscope.*
8. Continue to section at 8-12 micron thickness. Gently transfer each section to room temperature gelatin-coated slide by allowing section to melt onto slide.
9. Allow sections to adhere to slide at room temperature for at least 2 hours prior to immunostaining. *Pause point: slides may stored at -20 °C for up to a month. In this case, slides should be brought to room temperature prior to beginning immunostaining.*

Immunostaining

10. Circle area of interest on slides with hydrophobic PAP pen. This will form a well to hold block and antibody solutions. Be careful not to touch sections. Rehydrate slides in PBTD, [0.1% Tween-20, 1% DMSO in 1 X PBS] at room temperature for 2-3 min in Coplin Jar.
11. Remove slides from PBTD, drain excess off slide and place slides in humid chamber. *From this point forward, it is critical to not let the slides dry.*
12. Gently pipette ~200-300 microliters of Block [5% NGS in PBTD] onto slides (Note: solution volume depends on how large area of interest).
13. Incubate at room temperature for 1-2 hours.
14. Remove Block from slides by draining off excess onto kimwipes
15. Add ~200-300 microliters of primary antibody diluted in block.

16. Incubate in humid chamber overnight at 4 °C.
17. Remove primary antibody by rinsing slides in PBTD 3 times at room temperature for 10 minutes each.
18. Drain excess PBTD, return slides to humid chamber and place 200-300 microliters of appropriate concentration of secondary antibody diluted in block.
19. Incubate at room temperature for 1-2 hours.
20. Remove secondary antibody by rinsing slides in PBTD 3 times at room temperature for 10 minutes each.
21. Drain as much PBTD from slide as possible. Add one drop of Vectashield mounting medium directly onto sections.
22. Carefully place no. 1 thickness coverslip on slide. Allow Vectashield to harden at room temperature for at least 3 hours before imaging. *Pause point; may place slides in 4°C for up to 1 week until ready to image.*
23. Image on confocal or fluorescent microscope (**Figure 3**).

Acknowledgements:

This protocol describes methods originally developed in John E. Dowling's laboratory at Harvard University. We thank Michael Ness for technical support. Research in J.M.G.'s lab is supported by grants from the American Health Assistance Foundation Macular Degeneration Research Program, the Knights Templar Eye Foundation and the Retina Research Foundation.

References

Gross JM, Perkins BD, Amsterdam A, Egana A, Darland TD, Matsui JI, Sciascia S, Hopkins N and JE Dowling. "Identification of Zebrafish Insertional Mutants With Defects in Visual System Development and Function". *Genetics* 170: 245-261.

Figure Legends:

Figure 1: Zebrafish embryos aligned in cryoblock.

Figure 2: Adult zebrafish eyes aligned in cryoblock.

Figure 3: Retinal section from 4 days post fertilization embryo stained with Zpr-1, a cone cell marker (blue), and PKC, a bipolar cell marker (red). Five 1 um optical z-sections were taken through the eye using a Zeiss PASCAL Confocal microscope and images were recombined into a composite z-stack with Zeiss LSM Imaging Software.

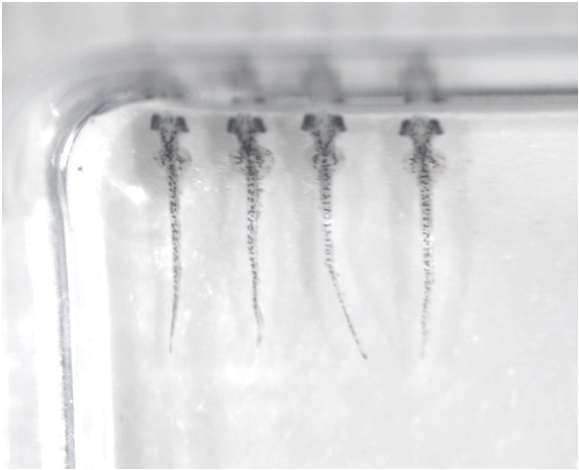


FIGURE 1

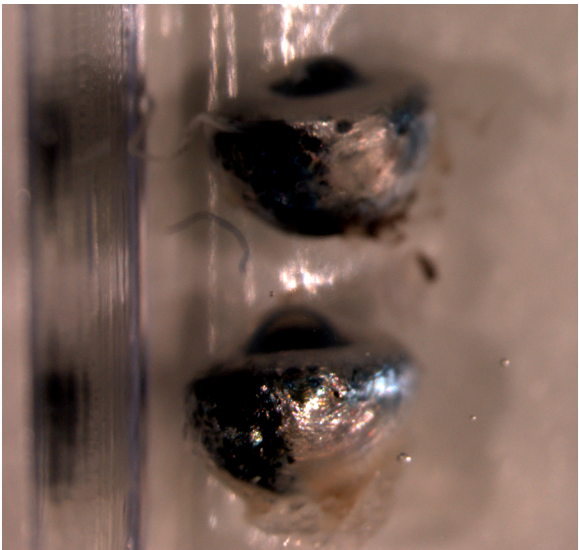


FIGURE 2

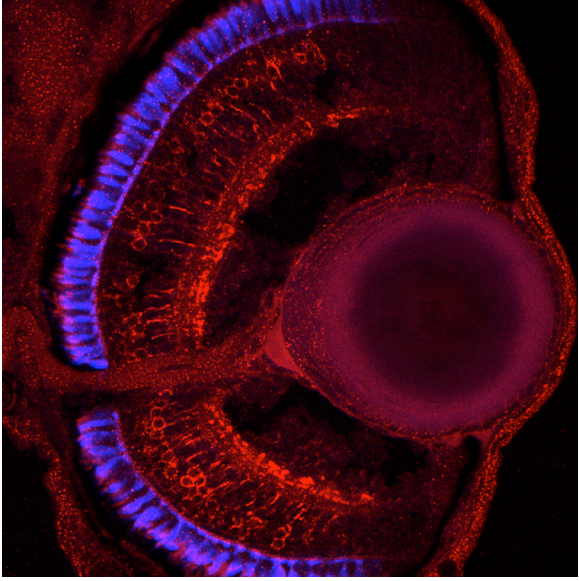


FIGURE 3