

Whole-mount Immunohistochemistry of Adult Zebrafish Retina for Advanced Imaging

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[Abstract] Immunohistochemistry is a widely used technique to examine the expression and subcellular localization of proteins. This technique relies on the specificity of antibodies and requires adequate penetration of antibodies into tissues. The latter is especially challenging for thick specimens, such as embryos and other whole-mount preparations. Here we describe an improved method of immunohistochemistry for retinal whole-mount preparations. We report that a cocktail of three reagents, Triton X-100, Tween-20, and DMSO, in blocking and antibody dilution buffers strongly enhances immunolabeling in whole-mount retinas from adult zebrafish. In addition, we establish that in whole retinal tissues, a classic epitope retrieval method, based on citrate buffer, is effective for immunolabeling membrane-associated proteins. Overall, this simple modification allows precise and reproducible immunolabeling of proteins in retinal whole-mounts.

Keywords: Photoreceptor, Müller glia, Visual system, Antibody staining, Microscopy

[Background] To understand complex biological processes, morphological and histological analyses allow for practical qualitative and quantitative approaches. Immunohistochemistry is a robust technique used to visualize the expression and localization of intra- and extra-cellular proteins. Although conventional histological sectioning offers high resolution images of immunostained proteins, sectioning 3-dimensional tissues results in poor preservation of tissue architecture. In sections, complete cellular structures and a complete understanding of the 3-dimensional distribution of proteins are not readily appreciated. In contrast, whole-mount preparations provide large volume 3D information, including intact cellular structures and the spatial relationships of cells and molecules in a complex tissue. However, adapting immunohistochemistry for use with intact tissues often results in poor permeability of antibodies, resulting in incomplete labeling, and a high degree of non-specific background.

In the retina, the cell body of the radial Müller glia spans the entire thickness of the retina (Bringmann *et al.*, 2006). Microglia, the innate immune cells in the central nervous system, with a small cell body and ramified processes, are distributed throughout the retinal parenchyma (Li *et al.*, 2015; Silverman and Wong, 2018). In response to neuronal damage or death, Müller glia and microglia undergo significant structural remodelling. Characterizing the morphology of these two cell types provides a readout the local environment surrounding each cell type and the overall health of the retina (Nagashima *et al.*, 2020; Silva *et al.*, 2020).

Detergents and organic solvents are common reagents that efficiently break down the permeability barriers of cells and nuclei (Jamur and Oliver, 2010). Triton X-100 and Tween-20 are widely used non-

ionic detergents, which perturb the phospholipid bilayer structure of biological membranes (Kalipatnapu and Chattopadhyay, 2005; Koley and Bard, 2010; Cheng *et al.* 2019). Although dimethyl sulfoxide (DMSO), a polar organic solvent, or acetone, a common fixative, has been shown to enhance permeability of cell membranes (de Ménorval *et al.*, 2012), its utility in immunohistochemical procedures is less common. Most procedures select a single reagent of detergents or solvents for permeabilization, and the efficiency of combining more than two chemicals is not well documented.

Here we describe an improved method of whole-mount immunohistochemistry. We report that a cocktail of three reagents, Triton X-100, Tween-20, and DMSO diluted in blocking and antibody dilution buffers dramatically enhances immunolabeling in whole-mount retinas. We also report that a classic citrate buffer based epitope retrieval method (Shi *et al.*, 1993) is effective for immunolabeling membrane associated proteins. For completeness, we also include a detailed protocol for isolating whole retinas from zebrafish.

Materials and Reagents

A. Animals

Adult zebrafish (Danio Rerio, Zebrafish International Resource Center, Eugene, OR, 4 to 12 months)

B. Materials

1. Colorfrost Plus Microscope Slides (Fisher Scientific, catalog number: 12-550-18)
2. Cover Glass, Rectangle No. 1½, 24 x 50 mm (Corning, catalog number: 2980-245)
3. 1.5 ml Microcentrifuge Tubes, Free of detectable RNase, DNase, DNA & pyrogens (USA Scientific, catalog number: 1615-5500)
4. 1 ml Syringe without needle (BD, REF 309659)
5. ProLong Gold Antifade Mountant (hard-set; ThermoFisher Scientific, catalog number: P36930)

C. Reagents

Tissue preparation and fixation

1. Tricaine-S/MS-222 (Syndel, Ferndale, WA)
2. Sodium Bicarbonate (Sigma-Aldrich, catalog number: S5761-500G)
3. Paraformaldehyde (SPI Supplies, catalog number: 02615-AB)
4. Sodium Hydroxide/NaOH pellets (Sigma-Aldrich, catalog number: S-0899)
5. Sodium Phosphate Monobasic Monohydrate/NaH₂PO₄·H₂O (Millipore Sigma, catalog number: SX0710-3)
6. Sodium Phosphate Dibasic/Na₂HPO₄ (Sigma-Aldrich, catalog number: S7907-1KG)
7. Sucrose (Sigma-Aldrich, catalog number: S9378-1KG)
8. 10x Anesthesia/Tricaine stock solution (see Recipes)
9. Anesthesia/Tricaine working solution (see Recipes)
10. 1.0% Sodium Bicarbonate solution (see Recipes)

11. 40% paraformaldehyde stock (see Recipes)
12. 4% paraformaldehyde in 0.1 M Phosphate buffer with 5% sucrose (see Recipes)
13. 10x Phosphate Buffer stock (1.0 M, pH 7.4) (see Recipes)
14. 0.1 M Phosphate buffer with 5% sucrose (see Recipes)

Immunohistochemistry

1. Sodium Citrate Dihydrate/ $C_6H_5Na_3O_7 \cdot 2H_2O$ (Fisher Scientific, catalog number: BP327-500)
2. Tween 20 (Fisher Scientific, catalog number: BP337-500)
3. Hydrochloric Acid/HCl (Fisher Scientific, catalog number: A144-500)
4. Sodium Phosphate Monobasic Monohydrate/ $NaH_2PO_4 \cdot H_2O$ (Millipore Sigma, catalog number: SX0710-3)
5. Sodium Phosphate Dibasic/ Na_2HPO_4 (Sigma-Aldrich, catalog number: S7907-1KG)
6. Sodium Chloride/NaCl (Sigma-Aldrich, catalog number: S7653)
7. Potassium Chloride/KCl (Sigma-Aldrich, catalog number: P3911-500G)
8. Triton X-100 (Sigma-Aldrich, catalog number: T9284-500ML)
9. Sodium Azide (Sigma-Aldrich, catalog number: S2002-100G)
10. Dimethyl Sulfoxide (Sigma-Aldrich, catalog number: D8418-100ML)
11. Goat Serum Donor Herd (Sigma-Aldrich, catalog number: G6767-500mL)
12. Anti-mCherry antibody (rabbit polyclonal, Abcam, catalog number: 167453)
13. Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, catalog number: A21245)
14. Mouse monoclonal anti-ZO1 (ThermoFisher Scientific, ZO1-1A12, catalog number: 33-9100)
15. Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (Invitrogen, catalog number: A21422)
16. Sodium Citrate Buffer (see Recipes)
17. 10x Phosphate Buffered Saline (PBS, pH 7.4) stock (see Recipes)
18. PBS (see Recipes)
19. PBS with 0.5% Triton X-100 (see Recipes)
20. 10x PBS with 1% sodium azide (see Recipes)
21. Whole-mount IHC blocking buffer (see Recipes)
22. Whole-mount IHC dilution buffer (see Recipes)
23. Whole-mount IHC washing buffer (see Recipes)

Equipment

1. Scissors, VANNAS, 8 cm STR (G) (World Precision Instruments, model: 14003-G)
2. Tweezers Dumont #5 INOX (World Precision Instruments, model: 501985)
3. Leica 205FA Stereomicroscope (Leica Microsystems)
4. Fisher brand Isotemp Stirring Hotplate (ThermoFisher Scientific, model: SP88857200)

5. Rocker
6. 1,500 ml beaker (PYREX, model: CE-BEAK1L)
7. LidLocks Microcentrifuge Tube Locks (VWR International, model: 14229-941)
8. Round Microcentrifuge Floating Bubble Rack (VWR International, model: 60-86-342)
9. Spoon Knife, Double Bevel, Angled 3.3 Blade (Hilcovision, model: 625-0743061-06)
10. 15 ml Conical Tube (FALCON, model: 352097)
11. Transfer Pipets (Fisher Scientific, model: 13-711-7M)
12. Injection Needle with LuerLok Syringe 1 ml 30G ½ (BD, model: 305778)
13. Zeiss AxiolImage ZI Epifluorescent Microscope (Carl Zeiss MicroImaging LLC)
14. Fisherbrand Nutating Mixers-Fixed speed (Fisher Scientific, model: 88-861-041)

Software

1. ImageJ
2. Adobe Photoshop CC 2019 (Adobe Systems)

Procedure

A. Retinal dissection and fixation

1. Dark adapt animals for at least 3 h prior to dissecting retinas.

Note: Dark adaptation is crucial to separate neural retina from the retinal pigment epithelium. However, this step can be omitted when staining synaptic components, as dark adaptation can induce rapid changes in synaptic structure.

2. In dark room under red illumination, sacrifice fish by immersion in ice cold water and cervical transection.
3. Using stereomicroscope, microscissors and tweezers, carefully insert microscissors into the back of the globe to visualize optic nerve (Figure 1, arrows). Cut the optic nerve, gently remove the globe, and place it on paper towel (Figure 2).

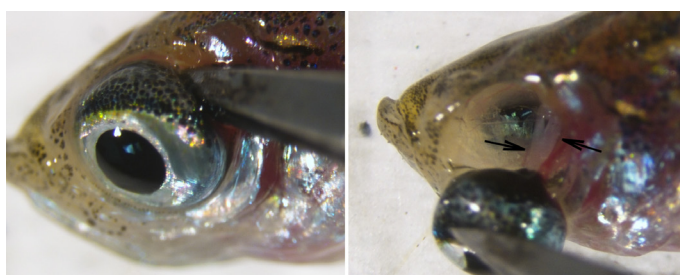


Figure 1. Enucleating the eye globe

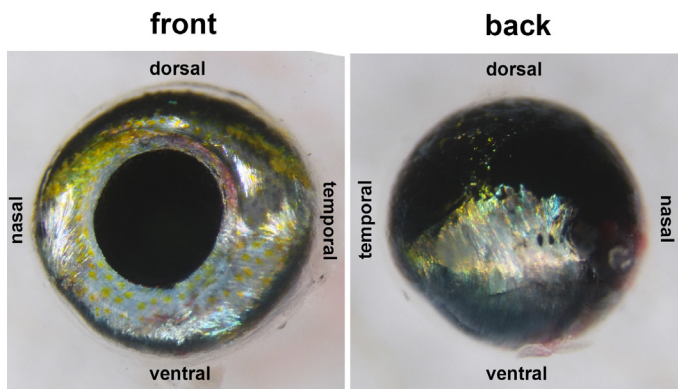


Figure 2. Front and back views of dissected globe

Note: For better separation of neural retina and the retinal pigment epithelium, avoid bright lights. Dim illumination is acceptable.

4. Using 30 G $\frac{1}{2}$ Injection needle, make a small hole ventrally at the choroid fissure (Figure 3, red circle).

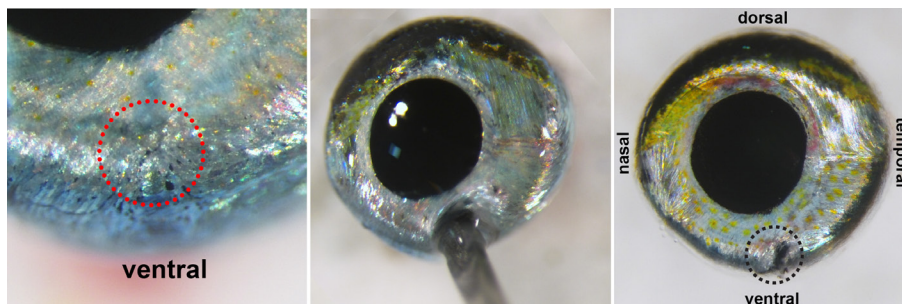


Figure 3. Globe with ventral hole

Note: If the choroid fissure is not visible, use other anatomical landmarks to distinguish the dorsal-ventral axis. The dorsal aspect of the eye has a greater amount of melanin/black pigments and fewer iridophores/shiny, silvery pigments, which are enriched ventrally (Figure 2).

5. With microscissors, extend the ventral hole about 5 mm toward the optic nerve head for orientation.
6. Cut circumferentially at the junction of the cornea and iris (Figure 4 left, red dashed line) with microscissors. Remove the entire anterior segment of the eye and lens (Figure 4).

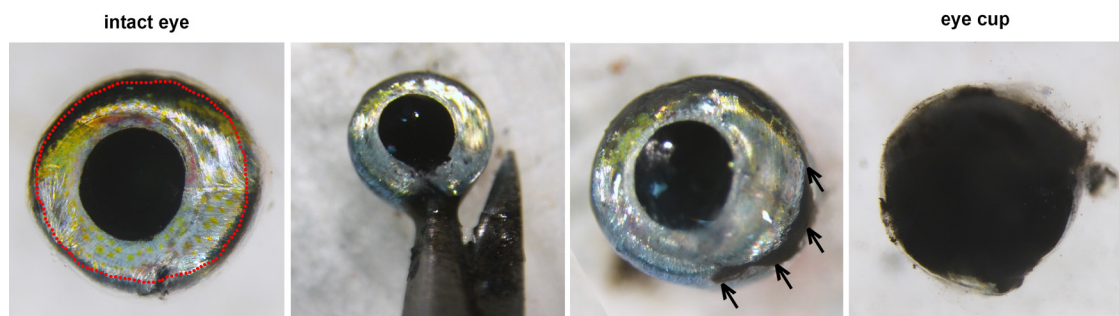


Figure 4. Removal of the anterior segment

7. Place the eye cup into PBS and gently separate the neural retina from retinal pigment epithelium by inserting micro tweezers between two layers. The neural retina is faintly pink and contrasts with the underlying retinal pigmented epithelium (Figure 5).

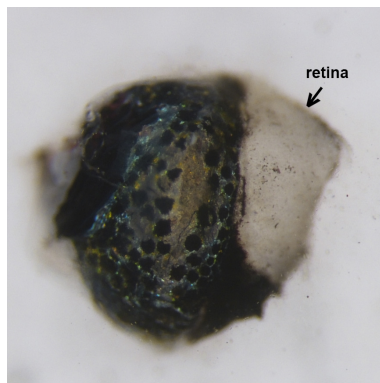


Figure 5. Lateral view of the eye cup in PBS

8. Using microsurgical blade, make short relaxation cuts from the margin toward the center of the retina at the nasal, temporal and dorsal quadrants (Figure 6). These cuts help to flatten retinas during mounting on the glass slides.

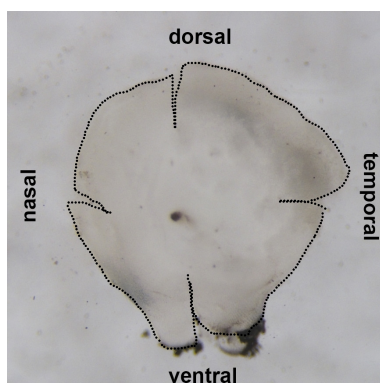


Figure 6. Dissected retina

9. Place individual retinas in 1.5 ml microcentrifuge tube containing 1 ml of 4% paraformaldehyde in 0.1 M Phosphate buffer (pH 7.4) with 5% sucrose, and fix overnight at 4 °C on rotator (Figure 7).

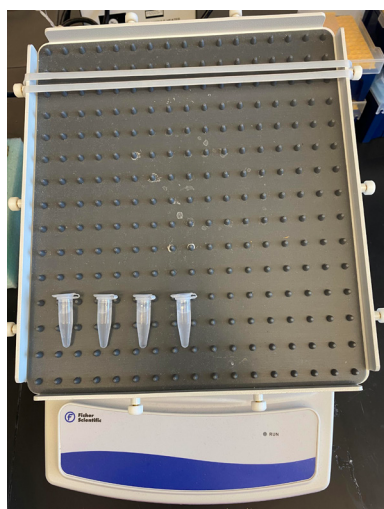


Figure 7. Fixation of retinas

10. The next day, rinse retinas with 1 ml of 5% sucrose in 0.1 M Phosphate buffer (pH 7.4) 3 times for 20 min each on rotator at room temperature.

B. Epitope retrieval (optional)

Note: This procedure is necessary if your antibody recognizes membrane-associated proteins, such as Zonula Occludens (Figure 13; Nagashima et al., 2020) and N-Cadherin (Nagashima et al., 2017).

1. Using hot plate, prepare a boiling water bath.
2. Exchange 5% sucrose in 0.1 M Phosphate buffer (pH 7.4) with 500 μ l of Sodium Citrate buffer.
3. Immerse centrifuge tubes in boiling water bath for 5 to 10 min (Figure 8).

Note: Install a LidLock on each microcentrifuge tube and use a floating rack.

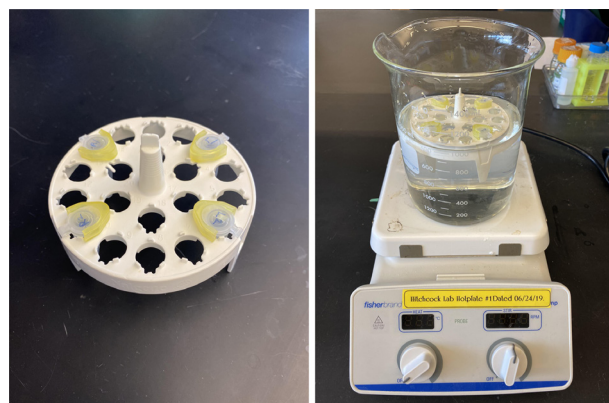


Figure 8. Epitope retrieval

4. Remove beaker from hot plate and let it cool down for 5 min. Do not remove microcentrifuge tubes from the beaker.
5. After 5 min, remove microcentrifuge tubes from the beaker and rinse retinas with 1 ml of PBS with 0.5% Triton X-100 for 10 min on rotator.

C. Immunohistochemistry

1. Remove buffer from the microcentrifuge tubes and add 500 μ l of Whole-mount IHC blocking buffer. Incubate retinas with Whole-mount IHC blocking buffer at room temperature for 2 h on rotator.
2. Dilute antibodies with Whole-mount IHC dilution buffer into optimized dilution (if unknown, start with 1:200). Make sure to centrifuge for 30 s before use.

Note: If you need double or triple labeling, mix all primary antibodies together. If your sample has transgenes, such as GFP or mCherry, we recommend using anti-GFP, anti-dsRed or anti-mCherry antibodies to amplify signals. Optimal dilutions for anti-GFP, anti-dsRed or anti-mCherry vary depending on the type of transgene, cell types transgenes are expressed, and the expression level. We recommend starting with 1:200 if optimized dilution is unknown. Anti-GFP, anti-dsRed, or anti-Cherry is necessary for retinas following epitope retrieval.

3. Incubate retinas with primary antibodies at room temperature overnight (NO agitation).
Note: Add at least 100 μ l of dilution buffer containing desired antibodies.
4. Rinse retinas with 1 ml of Whole-mount IHC washing buffer 20 min three times at room temperature.
5. Incubate retinas with secondary antibodies at room temperature overnight in dark.

Note: Add at least 100 μ l of dilution buffer containing desired antibodies with optimized dilution (if unknown, start with 1:200). If you need double or triple labeling, mix all secondary antibodies together. If possible, avoid green-fluorescent conjugate, such as Alexa Fluor 488, since green channel often shows higher background, especially after antigen retrieval.

6. Rinse retinas with 1 ml of Whole-mount IHC washing buffer 20 min three times at room temperature.

D. Mounting on slide glass

1. Using transfer pipette, transfer retina on a slide glass (Figure 9).

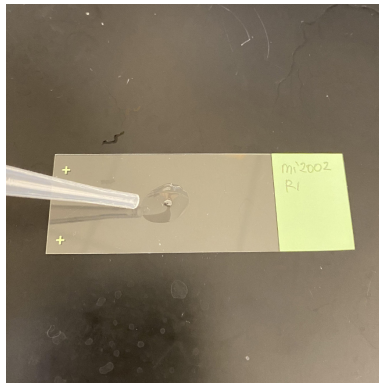


Figure 9. Transferred retina on a slide glass

2. Remove excess buffer with Kimwipe (DO NOT let retinas completely dry).
3. Using tweezer, clean up and orient retinas in photoreceptor side down. A large ventral cut (see Step A8) serves as a landmark for orientating the retina. Add 1 to 2 drops of Prolong Gold onto cover glass. Gently place the cover glass on the retina for mounting (Figure 10).

Note: Cure at least for 1 week at room temperature in the dark.

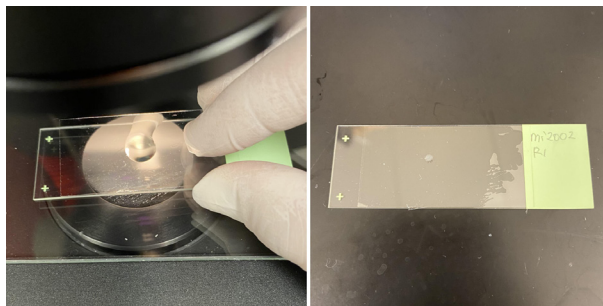


Figure 10. Mounting retina on a slide glass

Fixation

4% Paraformaldehyde in 0.1M Phosphate buffer (pH 7.4) with 5% sucrose
4°C overnight on rotator

Wash with 5% sucrose in 0.1M Phosphate buffer (pH 7.4)
3 times for 20 min each at room temperature on rotator

Epitope retrieval (Optional)

Incubate in Sodium Citrate Buffer
5 to 10 min in boiling water bath

Sodium Citrate Buffer (continued)
Cooling down on bench for 5 min

Wash with PBS with 0.5% Triton-X
10 min on rotator

Immunohistochemistry

Blocking with Whole-Mount IHC Blocking Buffer
Room temperature 2 hours on rotator

Primary Antibodies Incubation
Room temperature overnight (No agitation)

Wash with Whole-Mount IHC Washing Buffer
3 times for 20 min each at room temperature on rotator

Secondary Antibodies Incubation
Room temperature overnight (No agitation)

Wash with Whole-Mount IHC Washing Buffer
3 times for 20 min each at room temperature on rotator

Figure 11. Procedure of fixation and immunohistochemistry

Data analysis

Results and Discussions

Using the protocol described above (Figure 11), we successfully imaged *Tg(gfap:egfp)^{mi2002}* labeled Müller glia (Bernardos and Raymond, 2006; Nagashima *et al.*, 2017 and 2020), *Tg(mpeg1:mCherry)^{gl23}*-labeled microglia (Figure 12; Ellett *et al.*, 2011; Silva *et al.*, 2020), *Tg(-5.5sws1:egfp)^{kj9}*- or *Tg(trb2:tdTomato)*-labeled cone photoreceptors (Nagashima *et al.*, 2017; Nunley *et al.*, 2019), and *Tg(-3.7rho:egfp)^{kj2}*-labeled rod photoreceptors (Raymond *et al.*, 2014).

Optical sectioning is essential for imaging immunofluorescence in thick specimens. Confocal

microscopy eliminates out-of-focus signals and aids rapid three-dimensional reconstructions. Alternatively, we recommend epifluorescence microscopes equipped with structured illumination technology (Wu and Shroff, 2018) or computational clearing systems, which mathematically eliminate out-of-focus blur.

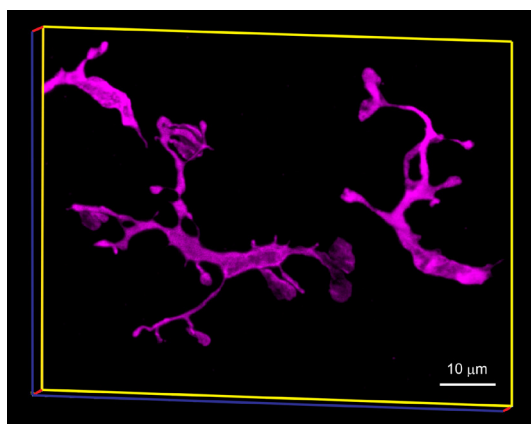


Figure 12. *Tg(mpeg1:mCherry)^{g/l23}*-labeled microglia in zebrafish retina

The transgene, *Tg(mpeg1:mCherry)^{g/l23}* (Ellett *et al.*, 2011), was immuno-labeled with rabbit polyclonal anti-mCherry (1:200) and Alexa Fluor 647 Goat anti-Rabbit antibodies (1:200). Image was captured using a Leica TCS SP5 confocal microscope (Leica Microsystems). ImageJ software (<https://imagej.nih.gov/ij/>) was used for 3D rendering.

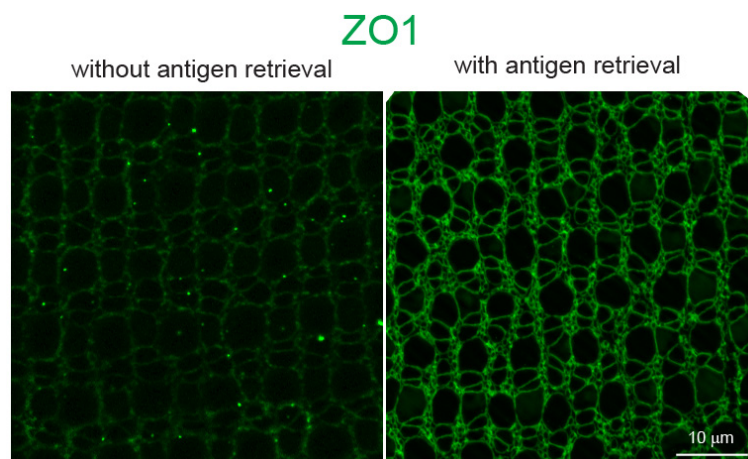


Figure 13. Comparison of zebrafish retinas immuno-labeled with ZO-1 without (left) and with (right) antigen retrieval

An integral membrane protein, ZO1, is immuno-labeled with mouse monoclonal anti-ZO1 (1:200) and Alexa Fluor 555 Goat anti-Mouse IgG antibodies (1:200) without (left) or with (right) antigen retrieval (Figure 13). Images were captured with a Zeiss AxioImage ZI Epifluorescent Microscope. To create maximum projection of the Z-stack series, Adobe Photoshop CC 2019 (Adobe Systems)

was used.

Recipes

1. 10x Anesthesia/Tricaine stock solution (10 mg/ml Tricaine methanesulfonate)
 - a. Add 1.0 g of Tricaine methanesulfonate (MS-222) to 100 ml of Mili-Q water
 - b. Adjust pH to 7.4 using 1.0% Sodium Bicarbonate solution
 - c. Make 2 ml aliquots and store frozen at -20 °C
2. Anesthesia/Tricaine working solution
Add 2 ml of Anesthesia/Tricaine stock solution to 100 ml zebrafish system water
3. 1.0% Sodium Bicarbonate solution
Add 1 g of Sodium Bicarbonate into 100 ml of Mili-Q water
4. 40% paraformaldehyde stock
 - a. Preheat 200 ml of Mili-Q water to 50-60 °C
 - b. Add 200 g of paraformaldehyde to preheated water
 - c. Stir well and add concentrated NaOH (NaOH pellets) until solution is clear
 - d. Bring up volume to 500 ml (adjustment of pH is not necessary)
 - e. Make 2 ml aliquots and store frozen
5. 4% paraformaldehyde in 0.1 M Phosphate buffer with 5% sucrose
 - a. Thaw 2 ml of 40% paraformaldehyde stock in/under hot water
 - b. Add 1 g of sucrose and 2 ml of 10x Phosphate Buffer stock to 16 ml of Mili-Q water
 - c. Add 2 ml of 40% paraformaldehyde stockStore at 4 °C, good for 2 weeks
6. 10x Phosphate buffer stock (1.0 M, pH 7.4)
 - a. Dissolve 13 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 55.75 g of Na_2HPO_4 in 350 ml of Mili-Q water
 - b. Adjust pH to 7.4 and bring volume to 500 ml with Mili-Q water
 - c. Autoclave for a long time storage
7. 0.1 M Phosphate buffer with 5% sucrose
 - a. Dissolve 25 g of sucrose in 400 ml of H_2O
 - b. Add 50 ml of 10x Phosphate buffer
 - c. Adjust pH to 7.4 if needed
 - d. Bring up volume to 500 ml with Mili-Q water
8. Sodium Citrate Buffer (10 mM Sodium Citrate with 0.05% Tween 20, pH 6.0)
 - a. Add 0.294 g of $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ to 100 ml of Mili-Q water
 - b. Adjust pH to 6.0 using 1 N HCl
 - c. Add 0.05 ml of Tween 20 and mix well
 - d. Store at room temperature for 3 months, or at 4 °C for longer storage
9. 10x Phosphate Buffered Saline (PBS, pH 7.4) stock
 - a. Add 2.76 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 11.36 g of Na_2HPO_4 , 87.6 g of NaCl, 1.87 g of KCl to 850 ml

of Mili-Q water

- b. Adjust pH to 7.4
- c. Bring to volume to 1 L

Each must be dissolved in solution before the next salt is added

10. PBS

- a. Add 100 ml of 10x PBS stock to 900 ml of Mili-Q water
- b. Adjust pH to 7.4

11. PBS with 0.5% Triton X-100

- a. Add 0.5 ml of Triton X-100 into 100 ml of PBS
- b. mix well until Triton X-100 is completely dissolved

12. 10x PBS with 1% sodium azide

Add 0.5 g of sodium azide into 50 ml of 1x PBS

13. Whole-mount IHC blocking buffer (10% Normal goat serum, 1% Tween-20, 1% Triton X-100, 1% DMSO in 0.1 M PBS with 0.1% sodium azide)

- a. Add 1.0 ml of 10x PBS with 1% sodium azide to 7.7 ml of Mili-Q water
- b. Using disposable syringe, add 0.1 ml of Tween-20 and 0.1 ml of Triton X-100 into PBS containing sodium azide (a)
- c. Add 100 μ l of DMSO
- d. Mix well until Tween-20 and Triton X-100 are completely dissolved
- e. Add 1 ml of Normal goat serum
- f. Store at 4 °C, good for 1 week

14. Whole-mount IHC dilution buffer (2% Normal goat serum, 1% Tween-20, 1% Triton X-100, 1% DMSO in 0.1M PBS with 0.1% sodium azide)

- a. Add 1.0 ml of 10x PBS with 1% sodium azide to 8.65 ml of Mili-Q water
- b. Using disposable syringe, add 0.1 ml of Tween-20 and 0.1 ml of Triton X-100 into PBS containing sodium azide (a)
- c. Add 100 μ l of DMSO
- d. Mix well until Tween-20 and Triton X-100 are completely dissolved
- e. Add 50 μ l of Normal goat serum
- f. Store at 4 °C, good for 1 week

15. Whole-mount IHC washing buffer (PBS with 1% Tween-20, 1% Triton X-100, 1% DMSO)

- a. Add 10 ml of 10X PBS to 87 ml of Mili-Q water
- b. Using disposable syringe, add 1 ml of Tween-20 and 1 ml of Triton X-100 into PBS
- c. Add 1 ml of DMSO
- d. Stir well until Tween-20 and Triton X-100 are completely dissolved
- e. Store at 4 °C, good for 1 week

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Competing interests

The authors declare no competing interests.

Ethics

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

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