Immunohistochemistry of the Zebrafish retina

Alison M. Ruf

Department of Biology

Honors Research Project

Submitted to

The Williams Honors College
The University of Akron

Approved:	Accepted:
Jordan Renna Date: 4/25/2022	Brian Bagatto Digitally signed by Brian Bagatto DN: cn=Brian Bagatto, o=University of Akron, ou=Department of Biology, email=bagatto@uakron.edu, c=US Date: 2022.04.25 16:46:05 -04'00' Date:
Honors Project Sponsor (signed)	Honors Department Advisor (signed)
Dr. Jordan Renna	Dr. Brian Bagatto
Honors Project Sponsor (printed)	Honors Department Advisor (printed)
Date: 4/25/22	Date: 4/25/22
Reader (signed)	Department Chair (signed)
Dr. David Modarelli	Dr. Steve Weaks
Reader (printed)	Department Chair (printed)
Date: 4/25/22	
Reader (signed)	
Dr. Rolando J.J. Ramirez	
Reader (printed)	

Immunohistochemistry of the Zebrafish retina

Alison Ruf

Sponsor: Dr. Jordan Renna, Ph.D

Williams Honors College Project - Spring 2022

Abstract

The retina is made up of many different essential parts that work together to allow for vision of many different species. Some of these crucial cells and proteins include ganglion cells, bipolar cells, photoreceptors, synaptic markers, such as VGLUT, and receptors that bind glutamate. The basis of this project is to use immunohistochemistry to identify these important aspects of the retina in zebrafish via monoclonal and polyclonal antibodies. The goal being to compare specific cells in a control group retina to an experimental group of zebrafish.

Introduction

The retina is located at the back of the eye and is the layer that contains photoreceptor cells. The most common photoreceptor cells are rods and cones, which detect different colors and light intensities, but another photoreceptor cell type is photosensitive retinal ganglion cells. The retina houses many other cells that help propagate the signals from the photoreceptor cells to the optic nerve, including bipolar cells, horizontal cells, amacrine cells, and ganglion cells. Proteins are also key in this mechanism, including synaptic markers, such as VGLUT, and receptors that bind the neurotransmitter

glutamate. All these cells work together to allow for accurate visual information to make it to the brain via the optic nerve.

Rods, cones, and other integrating types of neurons are all required for visual sensory processing. This transduction pathway starts when light hits the photoreceptor (rods/cones), which sends a signal to bipolar cells. Photoreceptor cells send this signal to bipolar cells by releasing glutamate, as at this synapse glutamate is inhibitory. Glutamate is released by photoreceptors onto bipolar cells in the dark, and this release is inhibited in the light, thus regulating the signal. Bipolar cells then synapse on amacrine cells and ganglion cells. Amacrine cells are found in the inner plexiform layer and serve to regulate the interconnections. Ganglion cells receive signals from bipolar cells via the release of glutamate, which is excitatory at this synapse rather than inhibitory. Glutamate is released when sending a signal and is inhibited when dark. The axons of the ganglion cells then form the optic nerve, which sends these signals to the brain via action potentials (Hill et. al., 2016). Due to these different cells and proteins that must work together for a functional retina, immunohistochemistry can be beneficial to identify what parts of the retina are affected in different disease models. Immunohistochemistry is a technique that uses primary antibodies and then a secondary antibody that binds to that primary antibody with a dye that is detectable via a florescence microscope. When selecting antibodies, the primary antibody needs to bind directly to the protein of interest and can be polyclonal or monoclonal, with each having advantages and disadvantages. Polyclonal antibodies result in more staining and because of that can lead to false positives by binding to unwanted sites, whereas

monoclonal antibodies have a much higher specificity resulting in a much weaker stain (Matos et. at., 2010). Pooled monoclonal antibodies result in both high levels of staining and a high specificity, but due to their low availability they are not as commonly used (Yaozhou et. at., 2020).

The purpose of immunohistochemistry is to see the exact location where specific antigens or proteins of interest are expressed in each tissue sample. This localization of specified proteins is crucial in fields dealing with the diagnostics, specifically infections or cancers (Matos et. at., 2010)

The purpose of this study was to find a reliable immunohistochemistry protocol for zebrafish and then to research and test specific antibodies that can bind intended proteins on the zebrafish retina. The best results of zebrafish immunohistochemistry came from a bio-protocol written by Mikiko Nagashima and Peter F. Hitchcock titled, Whole Mount Immunohistochemistry of Adult Zebrafish Retina for Advanced Imaging (Nagashima & Hitchcock, 2020). After verifying that this protocol could be replicated using mouse anti-TH antibodies, more specific antibodies could be tested. Mouse anti TH is a primary antibody that binds to tyrosine hydroxylase, the rate-limiting enzyme for the biosynthesis of the catecholamines. TH is present in some of the amacrine cells of the inner plexiform layer of the retina and dopamine is the neurotransmitter of these cells (Yang et al., 2018). Following the use of TH, other antibodies found to test include GluR2 a mouse monoclonal antibody that labels a recombinant fusion protein TrpE-GluR2, GluR4 a rabbit polyclonal antibody against a C-terminus peptide of rat GluR4

conjugated to BSA with glutaraldehyde, mGlur1a a rabbit polyclonal antibody against the C-terminal peptide of rat mGluR1a conjugated to KLH with glutaraldehyde and lastly NR2B another rabbit polyclonal antibody against a C-terminal fusion protein of the NMDAR2B (Klooster et al., 2009). The goal of this specific project was to find antibodies that work sufficiently to allow for more research, specifically answering a later question of specific cells present in a control group of zebrafish retina versus an experimental group of zebrafish.

Materials and Methods

This immunohistochemistry protocol was done on a dissected free-floating zebrafish retina. The retina was dissected in a lit environment within PBS solution. Once the eyecup was separated from the retinal pigment epithelium the immunohistochemistry could be conducted. This protocol is based on a bio-protocol written by Mikiko Nagashima and Peter F. Hitchcock (Nagashima & Hitchcock, 2020). The first step of this procedure is to fix the retina overnight. This is one major difference between the previous immunohistochemistry protocol attempted on zebrafish before this research. The retina is fixed in 1 mL of 4% PFA in 0.1 M PBS with 5% sucrose. This solution was made by first making a 30% sucrose stock solution (10.26g sucrose into 100mL of PBS) and then using 167 uL of that stock solution, 125 uL of 32% PFA, and 708 uL of PBS solution. After the retina was fixed overnight three washes are done, each for 20 minutes, in a 5% sucrose solution. Five percent sucrose solution was made using 167 uL of the previous stock solution and 833 uL of PBS. Following this washing step epitope retrieval is done. This step is required when staining proteins that are

membrane-associated or that are potentially membrane-associated. Epitope retrieval is done by using a boiling hot water bath to heat a free-floating retina in 500uL of sodium citrate buffer for five minutes (see appendix A). After the five minutes of boiling the retina must cool for five minutes before rinsing the retina in 1 mL of PBS with 0.5% Triton X-100 for 10 min on the rotator (10uL triton X-100 and 990uL PBS). After this wash, the retina is added to 500 uL of whole-mount IHC blocking buffer and incubated on the rotator at room temp for 2 hours (see appendix B). Following this wash, the primary antibody was added. The first antibody used to test this protocol was mouse anti TH, 2 uL mouse anti TH, and 250uL of whole-mount IHC dilution buffer (see appendix C). The retina was incubated with the primary antibody at room temperature overnight. After the retina was incubated with the primary antibody overnight the retina was rinsed with whole-mount IHC washing buffer three times each for 20 minutes (see appendix D). After the three washes are done, the retina is added to the secondary antibody, donkey anti-mouse 350 and 250 uL of whole-mount IHC dilution buffer and incubated overnight in the dark. On the fourth day following the secondary antibody incubation, the retia were rinsed with whole-mount IHC washing buffer three times for 20 minutes each at room temperature (see appendix D). After these washes the retina can be mounted, first by transferring the retina to a glass slide using a transfer pipette, removing excess buffer with a kimwipe, orienting the retina photoceptor side down, adding 1-2 drops of aqua mount onto cover glass, and gently place the cover glass on the retina for mounting. The retina must then cure for at least one week at room temp in the dark before images can be taken.

Results

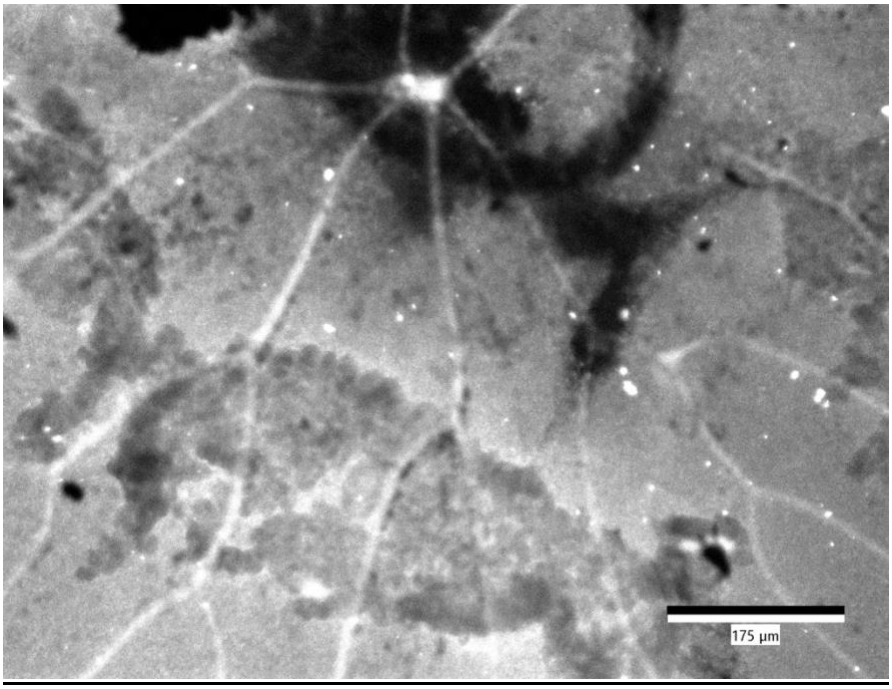

Figure 1: Stained image of a zebrafish retina, shows stained vasculature but does not show the intended staining of TH (tyrosine hydroxylase) – 10x magnification

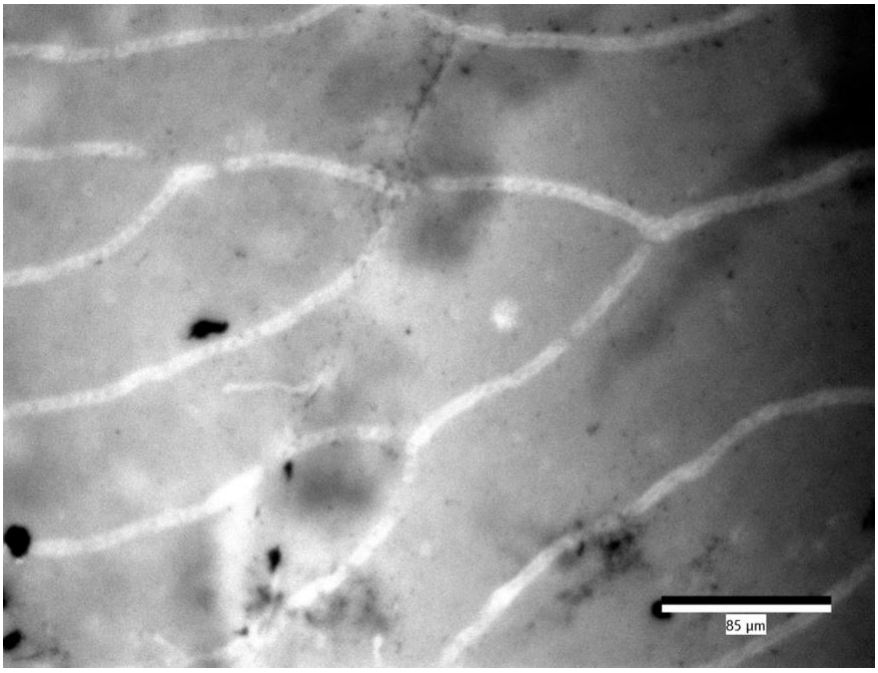

Figure 2: Stained image of a zebrafish retina, shows stained vasculature but does not show the intended staining of TH (tyrosine hydroxylase) -- 20x magnification

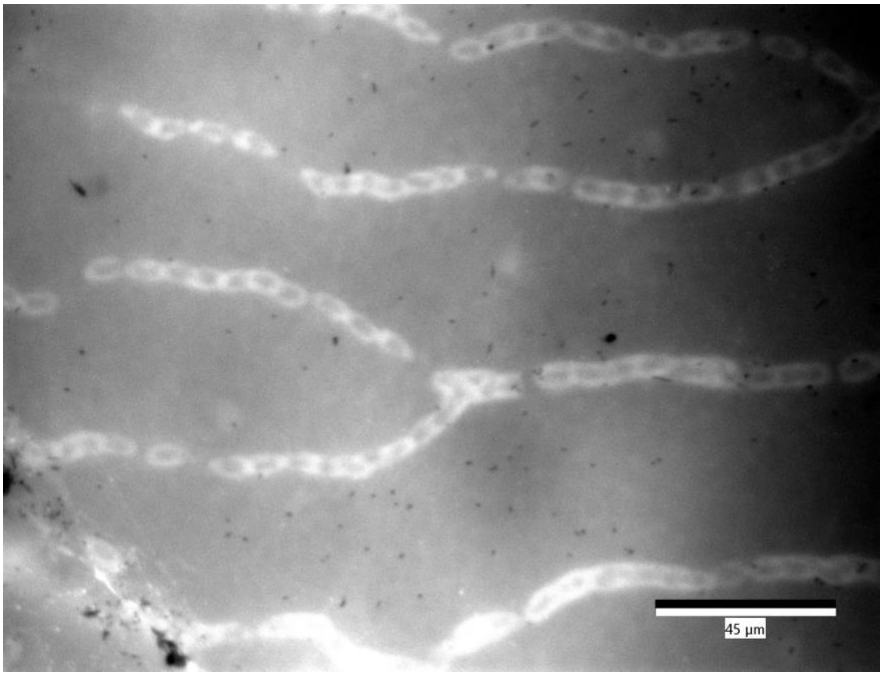

Figure 3: Stained image of a zebrafish retina, shows stained vasculature but does not show the intended staining of TH (tyrosine hydroxylase) -- 40x magnification

Discussion

This experiment did not have the resulting images expected from the staining that was done, rather figure 1, 2, and 3 show stained vasculature in the zebrafish retina **(figure 1, figure 2, and figure 3)**. The retina was stained with a primary antibody, mouse anti TH, and a secondary antibody, donkey anti-mouse 350, to stain tyrosine hydroxylase on the zebrafish retina, but no such staining is seen on the images. The staining should be seen where catecholamines and epinephrine cells are found. In the zebrafish retina that would be in the synapses of amacrine cells of the inner plexiform layer of the retina where dopamine, a catecholamine, is the neurotransmitter.

There is a multitude of reasons that this procedure may have not effectively stained the retina. First, using 350 (blue) secondary antibodies to stain small proteins is not the most effective due to not being able to see that staining on the back background. When I repeat this procedure, I will be using a red or green secondary antibody to avoid this issue in the future. Another possible problem is with the procedure itself. One major difference between this procedure and others in the field is how long the retina was fixed. Potentially the retina may have been in that solution for too long. The protocol states overnight but this can be a variety of time frames. Fixation is the process of preserving cells in a life-like state to allow for the preparation of thin stained sections. Extended fixation is detrimental to the retina because it leads to poor paraffin embedding leading to poor resistance to high-stress antigen retrieval techniques (Otali et. al., 2009). When this occurs, morphology is impaired, and potential desired protein of interest can be compromised. Reducing this fixation step to 12 hours could reduce these issues and allow staining to work.

Another differentiation from this protocol to the traditional immunohistochemistry protocols is the epitope retrieval step. This required the retina to be boiled in a sodium citrate buffer solution for five minutes. Although this boiling of the retina was done to allow staining of proteins that are membrane-associated it could have potentially denatured, become insoluble, and remained that way even after being cooled. This would explain why the antibodies were unable to bind to the protein of interest and no staining was seen.

The last possible problem is an experimental error, this was my first time researching, writing, and replicating an experimental procedure in the lab. Due to this experimental error, it is very probable along with these other potential issues with the protocol.

Once this immunohistochemistry protocol is perfected further antibodies can be used such as GluR2, GluR4, mGlur1a, and NR2B to stain more specific synaptic markers in the retina.

Works Cited

Hill, R. W., Wyse, G. A., & Anderson, M. (2016). *Animal physiology*. Sunderland, Massachusetts: Sinauer Associates, Inc.

Klooster, J., Yazulla, S., & Kamermans, M. (2009). Ultrastructural analysis of the glutamatergic system in the outer plexiform layer of zebrafish retina. *Journal of chemical neuroanatomy*, *37*(4), 254–265. https://doi.org/10.1016/j.jchemneu.2009.02.004

Matos, L. L., Trufelli, D. C., de Matos, M. G., & da Silva Pinhal, M. A. (2010). Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomarker insights*, *5*, 9–20. https://doi.org/10.4137/bmi.s2185

Nagashima, M., & Hitchcock, P. F. (2020). Whole-mount Immunohistochemistry of Adult Zebrafish Retina for Advanced Imaging. *Bio-protocol*, *10*(24), e3848. https://doi.org/10.21769/BioProtoc.3848

Otali, D., Stockard, C. R., Oelschlager, D. K., Wan, W., Manne, U., Watts, S. A., & Grizzle, W. E. (2009). Combined effects of formalin fixation and tissue processing on immunorecognition. *Biotechnic & histochemistry : official publication of the Biological Stain Commission*, *84*(5), 223–247. https://doi.org/10.3109/10520290903039094

Yang, F., Ma, H., & Ding, X. Q. (2018). Thyroid Hormone Signaling in Retinal Development, Survival, and Disease. *Vitamins and hormones*, *106*, 333–349. https://doi.org/10.1016/bs.vh.2017.05.001

Yaozhou Zhu, Y. Zhu, & Yun-Hwa Peggy Hsieh, Y. Peggy Hsieh. (2020). Effect of storage and processing on the immunodetectability of fish proteins using pooled monoclonal antibodies in ELISA and dot blot. *Food control, 125*, 107976. doi: 10.1016/j.foodcont.2021.107976

Appendix

A - Stock Solution of Sodium Citrate Buffer

1. Add 0.294 g of $C_6H_5Na_3O_7 \cdot 2H_2O$ to 100 ml of DI water
2. Adjust pH to 6.0 using 1 N HCl
3. Add 0.05 ml of Tween 20 and mix well
4. Store at room temperature for 3 months, or at 4 °C for longer storage

B - Stock Solution of Whole mount IHC Blocking Buffer

1. Start with 8.7 mL of 0.1M PBS
2. Using disposable syringe, add 0.1 ml of Tween-20 and 0.1 ml of Triton X-100 into PBS
3. Add 100 µl of DMSO
4. Mix well until Tween-20 and Triton X-100 are completely dissolved
5. Add 1 ml of Normal goat serum
6. Store at 4 °C, good for 1 week

C - Stock Solution of Whole mount IHC dilution Buffer

1. Start with 9.65 mL of 0.1M PBS
2. Using disposable syringe, add 0.1 ml of Tween-20 and 0.1 ml of Triton X-100 into PBS
3. Add 100 µl of DMSO
4. Mix well until Tween-20 and Triton X-100 are completely dissolved
5. Add 50 µl of Normal goat serum
6. Store at 4 °C, good for 1 week

D - Stock Solution of Whole mount IHC washing Buffer

1. Start with 97 mL of 0.1M PBS
2. Using disposable syringe, add 1 ml of Tween-20 and 1 ml of Triton X-100 into PBS
3. Add 1ml of DMSO
4. Stir well until Tween-20 and Triton X-100 are completely dissolved
5. Store at 4 °C, good for 1 week