OPTIC NERVE OF ZEBRAFISH (*DANIO RERIO*):

AN ANIMAL MODEL FOR HUMAN AGING

by

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ABSTRACT

The optic nerve is the cranial nerve that sends messages from the eyes to the brain and is part of the central nervous system (CNS). Astrocytes help maintain neuronal health within the CNS. In humans, senescence of astrocytes is thought to be a factor in aging-related diseases. Glial fibrillary acidic protein (GFAP, or in fish Gfap) is a protein uniquely expressed by astrocytes. Higher than normal expression of GFAP is an indicator of reactive astrocytosis, which in turn occurs as a consequence of injury or insult to the central nervous system. With an eye toward determining whether zebrafish are a suitable model for studying aging-related changes to the central nervous system, I hypothesized that increased expression of Gfap in the optic nerve of zebrafish correlates with aging of zebrafish. I also investigated p16-ARC, a protein that has been associated with aging-related diseases. Additionally, a study that observed optic nerve of human cadavers revealed that the diameter of the optic nerve was greater in aged persons than in younger persons. Therefore, I measured the optic nerve of the zebrafish to see if it increased in diameter as the fish aged. I used immunohistochemistry to examining Gfap and p16-ARC expression in fish aged 3 – 12 months, and I found labeling intensity increased as the fish aged. The diameter of the optic nerve increased as well. Taken together, these results suggest further investigations of zebrafish optic nerve are warranted to lend support to using it as a model system to understand aging in humans.

I. INTRODUCTION

Aging is defined as the deterioration of an organism's physiological functions, such as those required for survival and fertility, which change over time (Gilbert, 2000). According to data compiled by the National Institute of Health (NIH), the National Institute on Aging (NIA) and the World Health Organization (WHO), the percentage of the population comprising individuals sixty-five and older has increased and will continue to increase globally for the next 20-30 years (Figure 1, He et al., 2016). This increase can be attributed to many factors, including advances in our scientific knowledge and medical breakthroughs that allow for better treatment of certain curable diseases. It is known that aging is a risk factor for many pathologies. Age-related pathologies, including late-onset Alzheimer's disease, have risen in occurrence in recent years mostly due to the increase in the elderly population (Bhat et al, 2012).

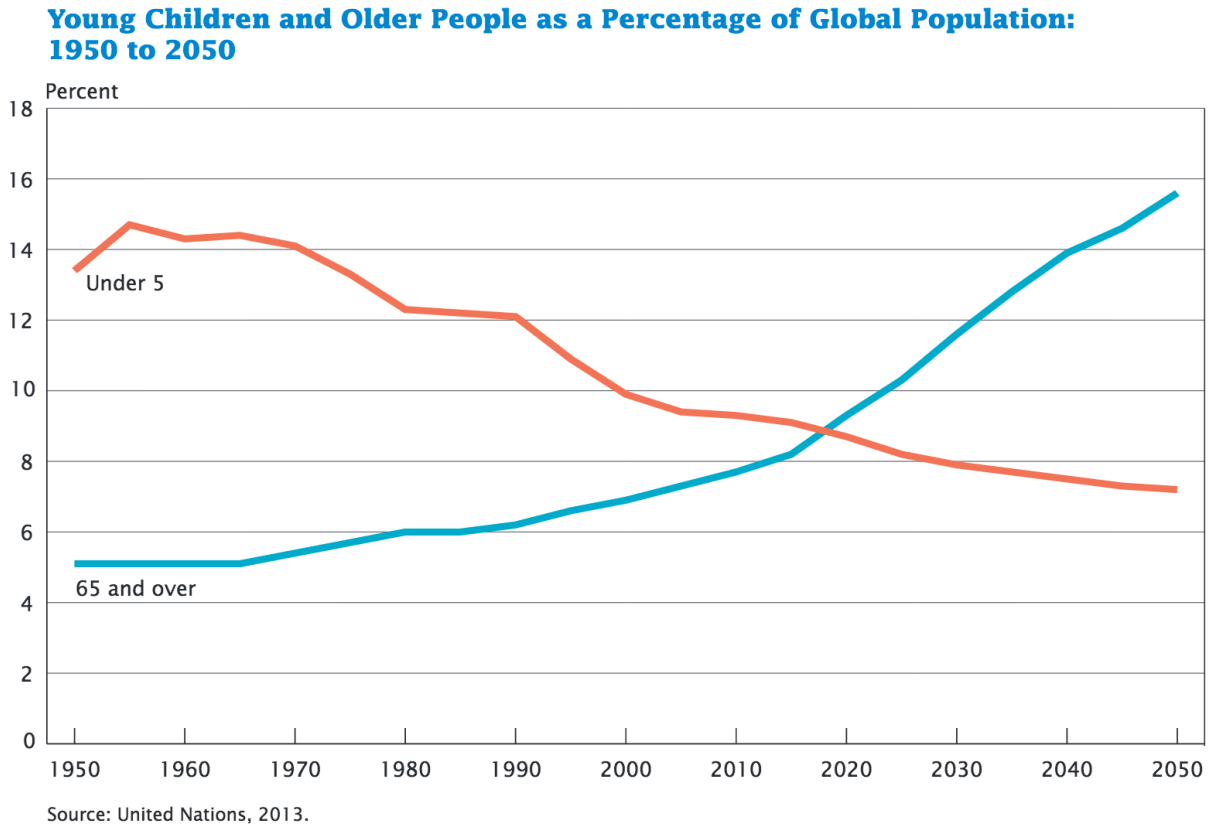

Figure 1: Data from the NIA illustrate the increasing population of people age 65 and over throughout the next several decades (He et al., 2016).

Alzheimer's disease generally affects older individuals. There are two classifications of Alzheimer's: (1) familial Alzheimer's, which is heritable, and (2) sporadic Alzheimer's, which is the more common and affects people after age 60-65 (Bird, 2015). In humans, cellular senescence, or deterioration with age, as well as other factors such as single-nucleotide polymorphisms (SNP's) have been suggested to be contributing factors leading elderly people to develop late-onset sporadic Alzheimer's disease (Bhat et al., 2012; Lesk, 2012). The disease affects mainly the central nervous system (CNS) and causes symptoms of dementia, including changes in emotions, critical reasoning, and memory loss (Bird, 2015).

Some studies propose that Alzheimer's-related molecular changes may affect different aspects of the human visual system, including the optic nerve (Chang et al., 2014; Tzekov and Mullan, 2014). Due to increased occurrence of this disease in recent years along with the trend in longer life spans, a major interest in the scientific community has been sparked that is focused on better understanding the processes involved in such age-related diseases. While many studies focus on the effects that the disease has on the brain, it is worthwhile to delve into the effects that the disease has on other parts of the CNS as well. The National Institutes of Health issued a call for the development of suitable animal models for better understanding the molecular changes that take place in age-related pathologies (National Institutes of Health, 2013). This thesis represents a response to that call and proposes the zebrafish as a suitable animal model.

Zebrafish as Model Organism

The practice of using zebrafish was initially popularized for better understanding of embryonic development. Zebrafish have the capacity to reproduce prolifically on a daily basis (on average about one to two hundred eggs per week), they have transparent embryos and they develop rapidly (Eisen, 1996; Gerhard, 2007) - major organs form within 24 hours after fertilization, hatching occurs after 3 days and sexual maturity within 3-4 months (Gerhard, 2007). Several researchers have proposed the use of zebrafish as a model for vertebrate aging (Gerhard, 2007; Kishi, 2014). As vertebrates zebrafish are more similar to humans than invertebrate models (e.g. *Caenorhabditis elegans*, *Drosophila melanogaster*, etc.), their genome is fully sequenced, they are inexpensive, they are feasible to manage in a laboratory setting, there are antibodies available for immunolabeling proteins of interest and they experience senescence (Newman et al., 2014). Zebrafish have a median lifespan of 36 to 42 months and a maximum lifespan of about 66 months (Gerhard et al., 2002; Keller and Murtha, 2004). Several studies have also investigated the possibility of using zebrafish as models related to aging, exercise and senescence (Gilbert et al., 2014; Kishi et al., 2003). For example, a study on the relationship between aging and exercise found that the turning frequency, which is used as an indicator of routine activity in zebrafish, decreased with age (Gilbert et al., 2014).

Astrocytes

Astrocytes, which are star-shaped, specialized glial cells that function as support cells in the CNS, play a major role in the pathological effects that affect the central nervous system (Sofroniew and Vinters, 2010). Bhat et al. (2012) suggested senescence

of astrocytes is directly correlated with the onset of Alzheimer's disease in humans (Bhat et al., 2012). Astrocytes express glial fibrillary acidic protein (GFAP), a type of intermediate filament, which surpasses the level of expression of another type of intermediate filament, vimentin, in the brains of humans as they develop (Middledorp and Hol, 2011). GFAP is also expressed in Mueller glia, astrocyte-like cells in the retina (Koke et al., 2010; Sofronview and Vinters, 2010). Studies performed by Nona (2005) using the goldfish visual system as a model showed that Mueller glial cells present in the optic nerve head also express Gfap.

Although fish astrocytes in the brain and spinal cord Gfap (Bernardos and Raymond, 2006), several studies suggest that astrocytes in the optic nerve of zebrafish do not express Gfap. Rather, glial cells in the optic nerve of zebrafish express cytokeratins instead (Conrad et al., 1998; Koke et al., 2010). In fact, Maggs and Scholes (1990) opine that fish optic nerve glial cells resemble neither radial glia nor astrocytes. Nevertheless, a member of the García lab observed expression of green fluorescent protein (GFP) in the optic nerve of a 30 month old, transgenic zebrafish engineered to express Gfap under the *gfap* promoter, suggesting that Gfap may be present in astrocytes of the aged optic nerve (unpublished observations, Savage and García). It was this observation that led me to ask if there is an increase in the expression of Gfap related to aging in zebrafish and if such a change could be considered a marker for senescence.

Reactive astrocytosis, also referred to as astrogliosis, is characterized by the altered gene expression, hypertrophy, and proliferation of astrocytes that occurs when the CNS responds to trauma, toxins, pathogens or neurodegenerative diseases (e.g., Alzheimer's, Parkinson's, or stroke; Sofronview, 2005; Sofronview and Vinters, 2010).

Astrocytes play several pivotal roles in the normal, uninjured CNS, such as maintaining brain homeostasis, providing cellular support to the axons and forming the blood-brain barrier. A study that focused on markers for reactive astrocytes following CNS trauma and ischemia found that there is an up-regulation of intermediate filament proteins, specifically GFAP and vimentin, (Pekny and Nilsson, 2005). Reactive astrocytes have the ability to adopt new functions that they do not normally perform after the CNS experiences trauma (Sofronview, 2005). A study comparing changes in the human optic nerves from cadavers of younger (18-22) and older (68-76) found that the number of astrocytes in the optic nerve of older subjects was increased (Cavallotti et al., 2002). These findings helped the researchers determine that the optic nerve in humans is quite sensitive to the aging process, making it a useful tool for understanding how aging affects the vision.

Optic Nerve Changes During Aging

The protein p16-ARC is a subunit of the Arp2/3 complex. The Arp2/3 (actin-related protein 2/3) complex comprises seven subunits that are involved in actin polymerization and formation of filamentous actin (Correa and Wales, 2012). Aside from its role in actin polymerization, Correa and Wales (2012) observed that p16-ARC has a role in the p38-MAPK pathway, activity of which is correlated with the onset of Alzheimer's disease. According to the model developed to propose p16-ARC's role as an actin remodeler within the p38-MAPK pathway, p16-ARC responds to the pathway's activation by remodeling actin and the cytoskeletal features in response to

neuroinflammation. It is important to note that this protein is still being further characterized to evaluate its role in premature senescence (Nie et al., 2014).

Overall, the focus of this thesis research was to observe the changes in the zebrafish optic nerve as zebrafish age with a particular focus on astrocytes. I hypothesize that changes in the zebrafish optic nerve illustrated by increased numbers of Gfap-positive astrocytes and an increased amount of Gfap fluorescence with other phenomena as well, including increases in the expression of p16-ARC and optic nerve diameter can be correlated with zebrafish aging. By collecting qualitative and quantitative data, I aim to characterize changes in the optic nerve as the zebrafish age in order to ask the question "Is zebrafish optic nerve a useful model for aging in humans?" Our research may advance biomedical research directed toward treating age-related diseases, including Alzheimer's disease.

II. MATERIALS AND METHODS

The Institutional Animal Care and Use Committee (IACUC) approved the protocols to perform this study. The IACUC protocol approval numbers are 1021_0826_19 for the molecular and morphological characterization of reactive astrocytes in zebrafish and 0626_0718_13 for the characterization of the optic nerve of zebrafish as an accessible model for aging research.

Immunohistochemistry and confocal microscopy were used to perform this investigation. Preliminary data collected by previous students in the García lab at various age points for the zebrafish served to guide our data collection and decisions about what ages of the zebrafish were to be observed. Data collected from the preliminary studies are not included in the analysis presented here as different antibodies were used. The data collected using confocal microscopy focused on the immunolabeled Gfap in the astrocytes and p16-ARC of the zebrafish optic nerve.

Fish care and euthanization

Wild-type, AB strain zebrafish aged 3 (n = 3), 6 (n = 4), 9 (n = 4), 12 (n = 4), 18 (n = 1) and 21 (n = 1) months were obtained and used to test if there was a trend in expression of Gfap and p16-ARC. The 3, 6, 9 and 12-month fish were purchased from the Zebrafish International Resource Center; the 18 and 21 month old fish had been bred in house. The 3-12 month old fish were euthanized immediately upon arrival, and the tissues were fixed and prepared for experimental use. The 18 and 21-month fish were maintained in tanks with aerated freshwater circulating through an activated charcoal/filter system located in the Supple Science Building Room 272, were fed daily

and were conditioned on a 12-hour light/12-hour dark cycle. The fish were separated in tanks according to the approximate hatch date that was noted in the lab for the 18- and 21-month fish, respectively. When house-bred fish reached the target age, a minimum of four fish were euthanized in a solution containing 0.2% Finquel® tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington) and 0.2% sodium bicarbonate dissolved in tank water. This humane process of killing the fish usually took between 2-4 seconds; fish were confirmed dead based on cessation of opercular movement.

Fixation, Preservation and Dissection

Once the fish were dead, they were placed in 15 ml, disposable centrifuge tubes containing 4% paraformaldehyde solution freshly prepared in phosphate buffered saline (PBS) comprising NaCl [0.1370 M], KCl [0.0027 M], Na_2HPO_4 [0.0100 M] and KH_2PO_4 [0.0018 M]. Specimens were left overnight in order to fix the tissue. The next day, the fish were washed three times for 10-15 minutes for each wash in PBS solution. Following the wash step, the fish were measured (Figure 1, Appendix), their sex identified, and then they were placed on a stereomicroscope for dissection. The eyes and brain of the fish were removed from the body (Figure 2, Appendix), and the organs were placed in a 30% sucrose solution overnight in order to cryoprotect the tissue in preparation for sectioning.

Sectioning

The cryoprotected tissue was embedded in Tissue-Tek®, frozen and sectioned into 20 μm thick sections using a Shandon cryotome (Figure 3, Appendix). Sections were

then collected by adhering them to gelatin-coated coverslips. Sections that contained a visible optic nerve (as seen in Figure 4, Appendix) were used in immunolabeling experiments. Sections were stored at -20^{o}C until they were processed for immunolabeling according to the standard lab protocol (Koke et al., 2010).

Immunohistochemistry

Prior to the addition of the antibodies, a humid microenvironment was prepared by placing a 40 mm, plastic petri plate inside a 100 mm, plastic petri plate with water in the bottom plate (Figure 5A). Before beginning the experiment, coverslips that contained tissue sections that included optic nerve tissue were selected. Different petri plates were labeled according to the chosen samples being experimental or control. The ratio of experimental to control sections that were processed was usually about three to four experimental sections to one control section. The first solution that was added to the tissue samples was 20% non-fat dry milk (NFDM), prepared in 10 ml of PBS. NFDM blocks nonspecific binding of the primary and secondary antibodies. Once added to the tissue that was inside of the microenvironment, the solution was left sitting for 2 hours. After 2 hours, three washes using PBS solution were performed for 10 minutes per wash. The antibodies for immunolabeling Gfap were anti-GFAP [zrf-1] raised in mouse (1:200 dilution) as the primary antibody (Abcam, Cambridge, UK) and goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:300 dilution) as the secondary antibody (Thermo Scientific, Waltham, MA). The antibodies for immunolabeling p16-ARC were anti-p16-ARC raised in rabbit (1:50 dilution) as the primary antibody (Abcam, Cambridge, UK) and goat anti-rabbit IgG (H+L) Cy5 (1:300 dilution) as the secondary antibody (Thermo Scientific,

Waltham, MA). During the wash steps, the primary antibody solution was prepared. The solution was centrifuged for 10 minutes at 10,000 rpm using an Eppendorf Centrifuge 5415-R with a 45° fixed-angle rotor. Once the primary antibody solution was prepared, 100 μL of solution were applied to the experimental tissue samples, while the control tissue samples were left in 100 μL of PBS solution. After incubating 2 hours at room temperature, three washes using PBS solution were performed for 10 minutes per wash. Next, the secondary antibody solution was prepared. The solution was centrifuged for 10 minutes at 10,000 rpm using the Eppendorf tabletop centrifuge. Once the secondary antibody solution was prepared, 100 μL of solution was applied to all of the tissue samples, both experimentals and controls. After 2 hours at room temperature, three washes using PBS solution were performed for 10 minutes per wash. Lastly, a solution containing 20 μL of Hoechst solution and 980 μL of PBS solution for a total of 1000 μL solution (1:50 dilution) was prepared. The solution was centrifuged for 10 minutes at 10,000 rpm using the Eppendorf centrifuge. Once the Hoechst solution, which is used for labeling nuclei within the optic nerve, was prepared, 100 μL of solution was applied to all of the tissue samples. After 30 minutes' incubation at room temperature, three washes using PBS solution were performed for 10 minutes per wash. The final step to prepare the tissue samples for imaging was to mount the coverslips onto slides with a small drop of anti-fade solution (90% glycerol prepared in PBS). Once adhered to the slides, the edges of the coverslips were sealed with nail polish.

Image Acquisition

Once IHC was complete, tissue samples were observed, and images were acquired using an Olympus FV1000 confocal laser-scanning microscope (Figure 6A). Firstly, the images were brought into focus to ensure that the tissue had not floated away during the IHC process. Images were acquired using the Planapo 60x oil immersion lens with a numerical aperture of 1.4. The settings were determined for each series of samples by first optimizing for the brightest point of fluorescence in an experimental sample from a 12-month-old fish. The confocal collection parameters were established based on each group that was being tested (i.e. one fish at each age group was tested at a time and the settings were optimized for the 12 month-old fish in each group). These laser settings were then used for the remainder of the samples in this experimental series to ensure that the data would be comparable. Prior to quantifying the data, the confocal images acquired were standardized for comparison by ensuring that all of the images were 75-optical slices thick in each z-stack image. Some optical sections had to be discarded if they did not meet at least 75-slice thickness in order to achieve the same thickness for every image. The step sizes were maintained at 0.38 nm.

Data Quantification and Analysis

In performing the data quantification, ImageJ software was used to determine the integrated density, which is a measure of the mean intensity within the selected region of interest (ROI) divided by the area of the ROI. The integrated density data is in units of pixel intensity per unit area. To determine whether increases in Gfap labeling was better attributed to upregulation of *gfap* as opposed to an increase in the number of astrocytes, I

calculated the integrated density per nucleus to test whether GFAP labeling increased in individual cells.

The optic nerve diameter measurements were obtained by taking three different diameter length measurements along the optic nerve and calculating an average value for the diameter in each fish sample. All quantitative data were analyzed using the R statistical package, employing ANOVA and post-hoc t-tests.

III. RESULTS

The expression of Gfap is evident in the optic nerve (Figure 2). Labeling is apparent in the optic nerve head of the 3-month old fish with no Gfap labeling in the distal area of the image in the optic nerve proper (Figure 2A). Little expression of Gfap is observed in the optic nerve of the 6-month fish (Figure 2C). The amount of Gfap labeling in the optic nerve of the 9-month fish is markedly increased (Figure 2E). The optic nerve of the 12-month fish shows intense Gfap labeling dispersed throughout the entire sample (Figure 2G). Labeling was quantified, and the data are compiled in Table 1 (Appendix). Overall, the amount of Gfap showed a trend toward increasing as the fish aged (Figure 3), but the increases were not statistically significant based on a one-way analysis of variance (ANOVA).

To investigate other senescence-related changes in the optic nerve, sections were labeled with anti-p16-ARC antibody. The expression of p16-ARC increased as the fish aged (Figure 4). No labeling of the p16-ARC protein was observed in any of the 3-month old fish (Figure 4A). The first appearance of p16-ARC is in the optic nerve of the 6-month old fish (Figure 4B). Labeling of p16-ARC is also observed in the optic nerve of the 9-month and 12-month old fish (Figure 4C and 4D). The compiled quantified data for all of the samples for p16-ARC can be observed for comparison (Table 2). The integrated density of p16-ARC labeling indicated a statistically significant ($p = 0.01$) increase in the amount of p16-ARC as the fish aged (Figure 5). Post-hoc t-tests revealed that the differences lay between 6- and 12-month old fish.

Lastly, mean optic nerve diameter increases with age of the zebrafish. The mean diameters of the zebrafish optic nerve for the 3, 6, 9 and 12-month age groups were

approximately 100 μm, 190 μm, 210 μm, and 260 μm, respectively (Figure 6). ANOVA indicated these diameters were statistically significantly different, and post-hoc analysis showed the differences lay between every group except 6- and 9-month old fish.

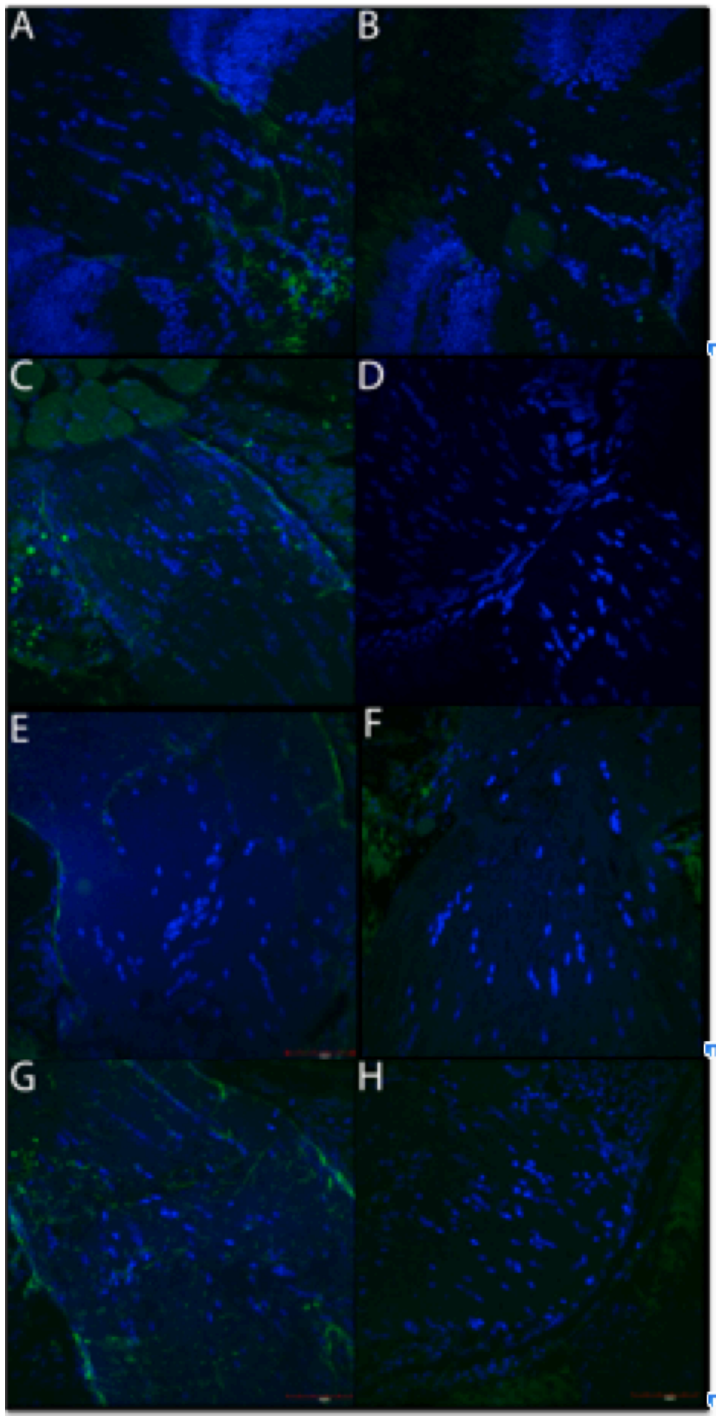

Figure 2. Confocal micrographs of optic nerve sections from 3- (A, B), 6- (C, D), 9- (E, F) and 12- (G, H) month old, wild-type zebrafish immunolabeled with anti-GFAP (green) and Hoechst for nuclei (blue).

Figure 2 continued: Experimental (A, C, E, and G) and negative control (B, D, F and H) images were acquired. The 3-month experimental section shows expected Gfap labeling in the optic nerve head, indicating that the labeling protocol was correctly executed, but no labeling in the optic nerve distal to the lamina cribrosa.

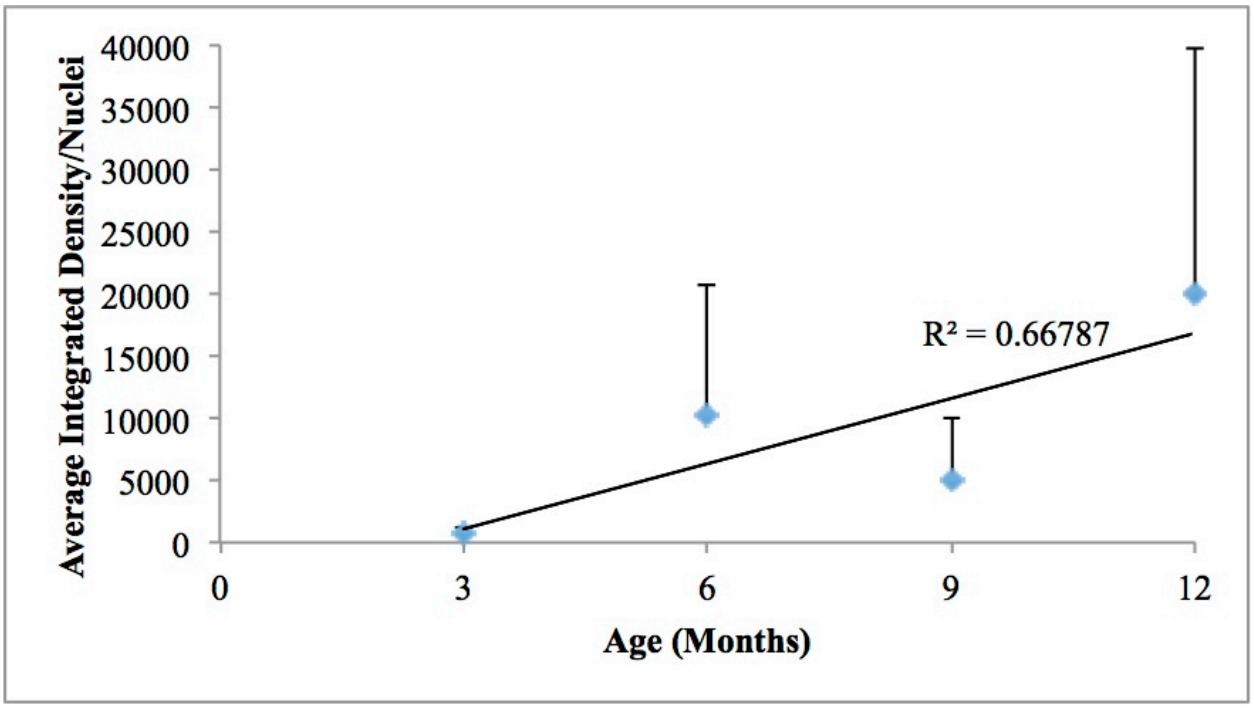

Figure 3: Average integrated density/nucleus of GFAP expression as a function of zebrafish age. Standard error bars represent variability in data. Although there appeared to be an upward trend in labeling intensity, data were not statistically significantly different using a p-value of 0.05.

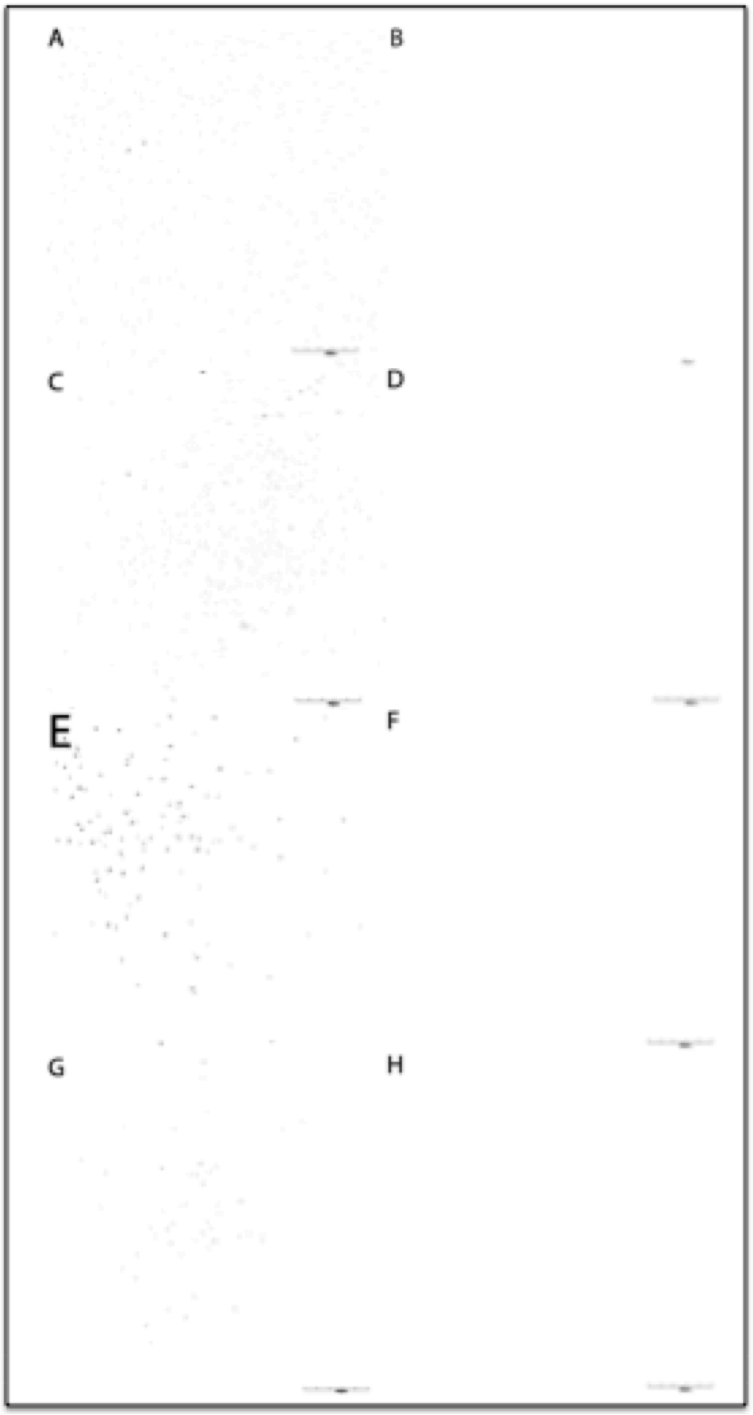

Figure 4: Optic nerve sections of 3, 6, 9 and 12 wild-type zebrafish immunolabeled with anti-p16-ARC raised in rabbit as the primary antibody and goat anti-rabbit IgG (H+L) Cy5 as the fluorescent dye (grey). The images were inverted to make labeling more easily visible. Experimental (A, C, E, G) and control images (B, D, F, H) were acquired.

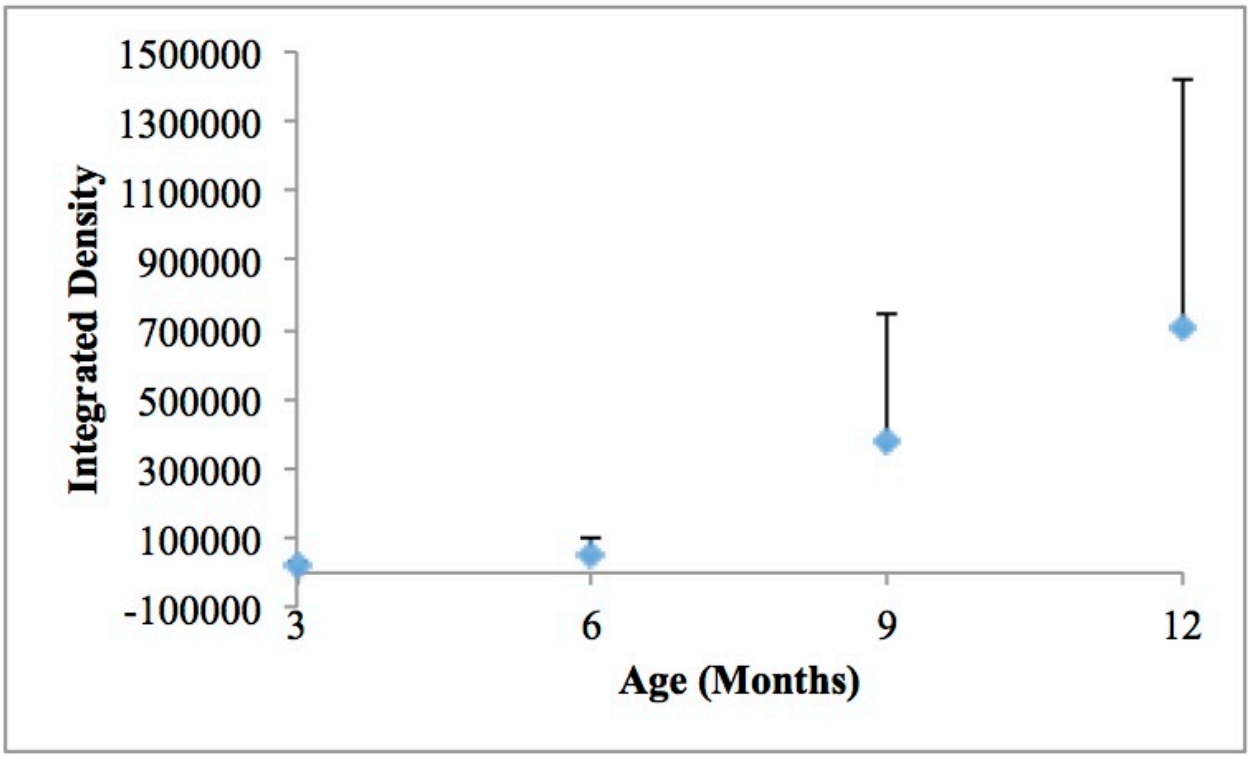

Figure 5: Average integrated density of p16-ARC expression as a function of zebrafish age. Standard error bars represent variability in data. R-squared value for the best-fit line (not shown) was 0.91347. Data were statistically significantly different ($p<0.05$), and the difference lay between 6- and 12-month old fish.

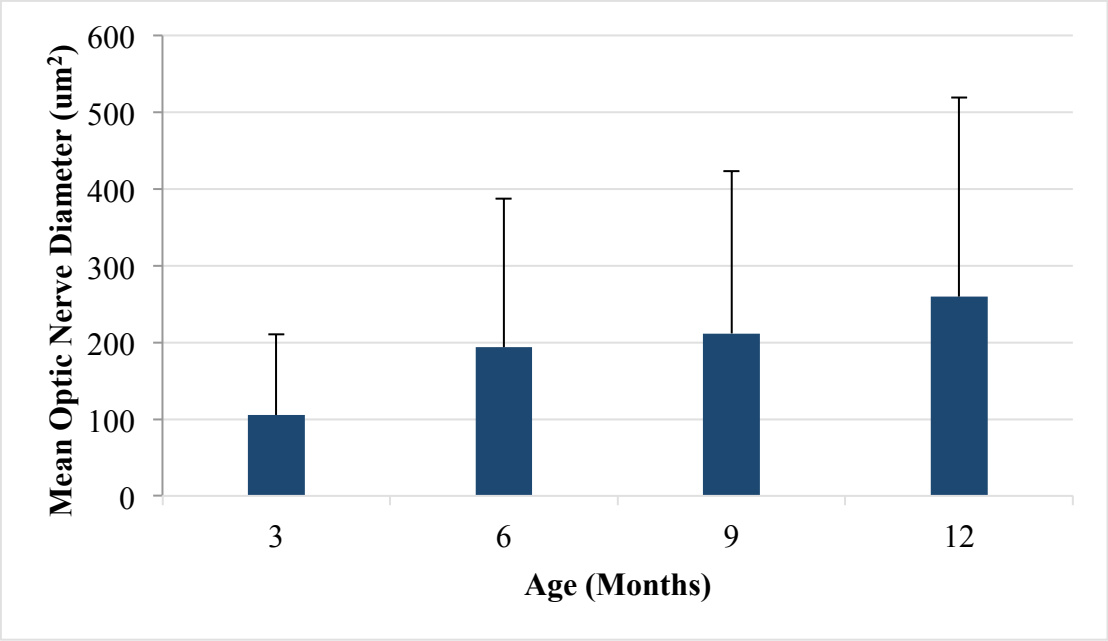

Figure 6: Changes in average optic nerve diameter as the zebrafish age. Standard error bars show the variability in the data. All means were statistically significantly different except 6- and 9-month olds ($p<0.05$).

IV. DISCUSSION

The overall goal of this research project was to test the hypothesis that Gfap and p16-ARC expression and diameter increase in the optic nerve of zebrafish as fish age. Our results suggest that the latter two factors increase with age in the optic nerve of zebrafish in a statistically significant manner while Gfap expression trends upward.

All of the 12-month fish displayed labeling for Gfap in their optic nerves. In contrast, not all of the younger fish showed labeling for Gfap. For the 6 and 9-month fish, only two out of four displayed significant Gfap expression, which may explain the statistical variability noted in Figure 3. Gfap labeling was observed in the retina and optic nerve head of the negative samples, but not in the optic nerve distal to the lamina cribrosa. The labeling in the retina and optic nerve head served as positive control as it is known that the Mueller glia in the retina express Gfap (Sofronview and Vinters, 2010; Koke et al., 2010). For the 3-month fish, weak Gfap expression was observed in two of the fish. However, the sample size was limited to three fish instead of four due to technical difficulties with sectioning the optic nerve tissue. When the best-fit line was applied to our statistical analysis, the slope was positive which suggests that there was an increase in Gfap expression (Figure 3); however, I was unable to detect a statistically significant difference. High levels of expression of Gfap are indicative of reactive astrocytosis (Pekny and Nilsson, 2005; Sofronview and Vinters, 2010; Liddelow et al., 2017); therefore, while the data in this limited data set cannot be said to support my hypothesis that age affects the optic nerve as a stressor, resulting in increased expression of Gfap as the zebrafish get older, they do suggest that increasing sample size could reveal a statistically significant trend.

The data in Table 2 suggests the amount of p16-ARC in the optic nerve of zebrafish increases with age as well. All sections immunolabeled with the antibody for p16-ARC showed expression of the protein in the optic nerves, but there was a significant increase noted as they reached older age, particularly from 6 months to 9 months (Figure 4). When the best-fit line was applied to our statistical analysis, the slope was positive which indicates that there was an increase in our data for p16-ARC expression as noted in Figure 5. Activation of p16-ARC within the p38-MAPK pathway is believed to function in actin remodeling and ultimately affects dendritic spine morphology, which is attributed to characteristics observed in individuals with Alzheimer's disease (Correa and Wales, 2012).

Lastly, our measurements of optic nerve diameter were intended to close the circle on factors that could contribute to supporting zebrafish as a model organism for human aging studies. As listed in the results section, the mean diameters of the zebrafish optic nerve for the 3, 6, 9 and 12-month age groups were approximately 100 um, 190 um, 210 um, and 260 um, respectively (Figure 6). The measurements display an obvious trend of increasing diameter of the optic nerves as the fish aged. These results suggest that when the zebrafish optic nerve experience a significant growth from 3 months to 6 months and then again from 9 month to 12 months. An interesting aspect of zebrafish is that the retina, as well as other nervous system tissues, grows throughout the fish's life, therefore, the increased diameter could reflect the addition of ganglion cells (among others) to the retina as the fish ages (Stenkamp, 2007).

Overall, our work has provided new insights into some of the novel functions that the astrocytes possess by examining their response to the aging process. Furthermore, in

considering future studies to be conducted it would be ideal to test other markers for senescence such as the cyclin-dependent kinase inhibitor, $p16^{INK4a}$, which previous studies have shown is marked by increased expression in response to senescence, and interleukin-6 (IL-6), which is a marker for neuroinflammation (Coppe et al., 2011).

APPENDIX SECTION

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Table

Figure 1A: Female zebrafish being measured prior to dissection.

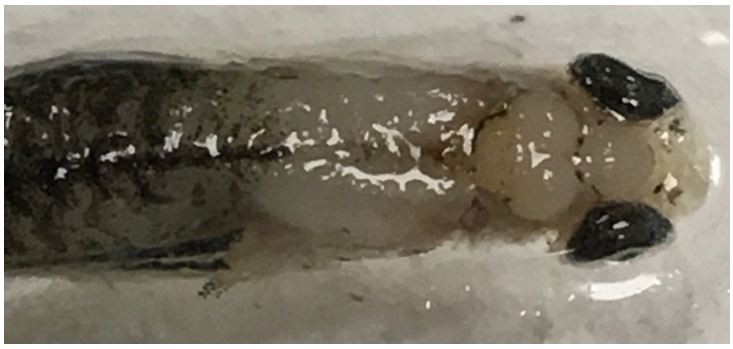
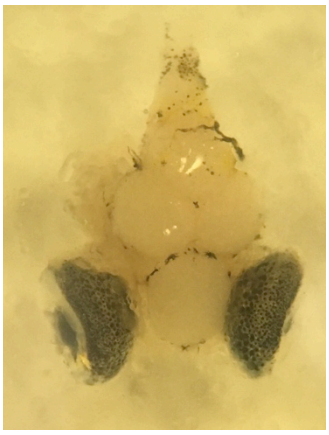
(a)  (b) 

Figure 2A: (a) Zebrafish during dissection before the eyes and brain are removed from the body. (b) Dissected eyes and brain.

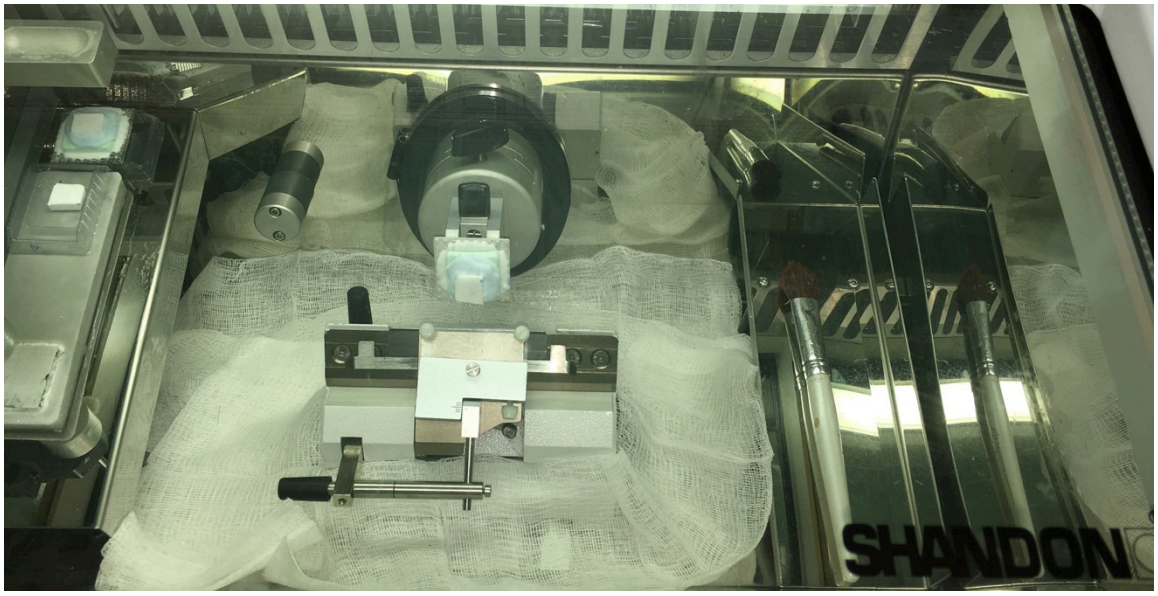


Figure 3A: Tissue block inside of the Shandon cryotome.

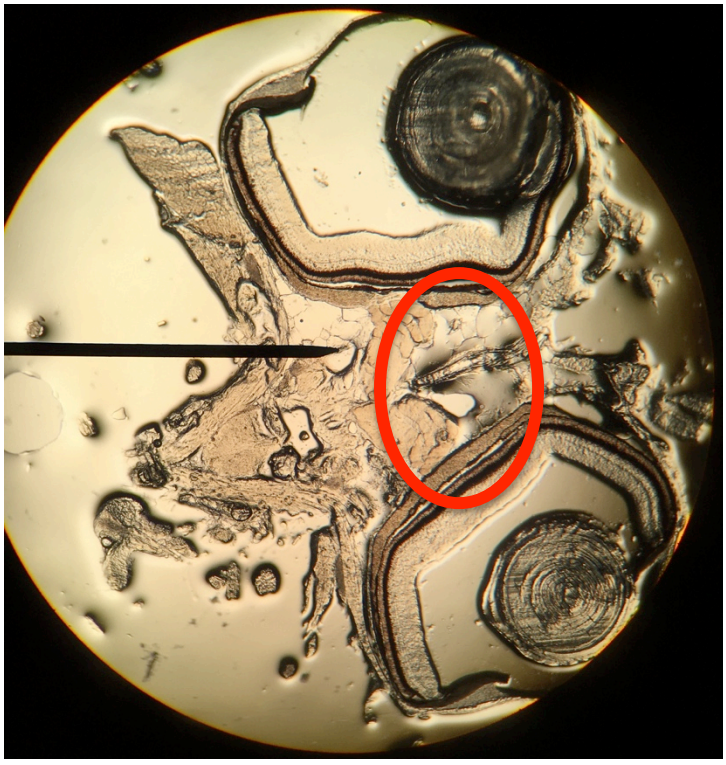


Figure 4A: Section of zebrafish eyes and brain that has visible optic nerves (red circle).

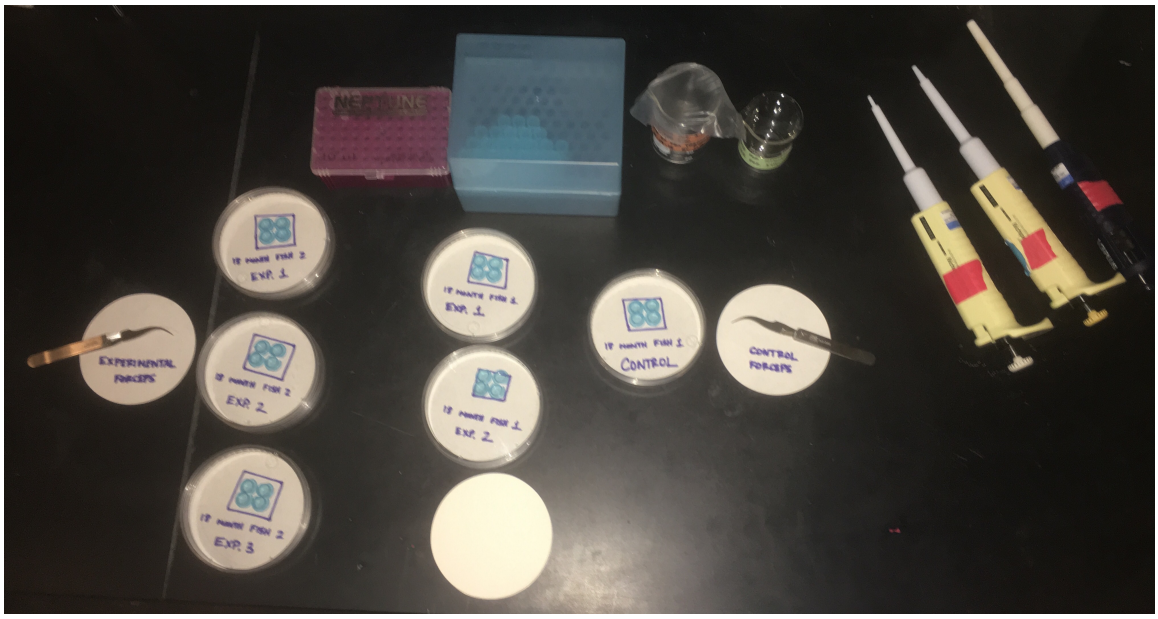


Figure 5A: Set-up prior to conducting immunohistochemistry experiment.

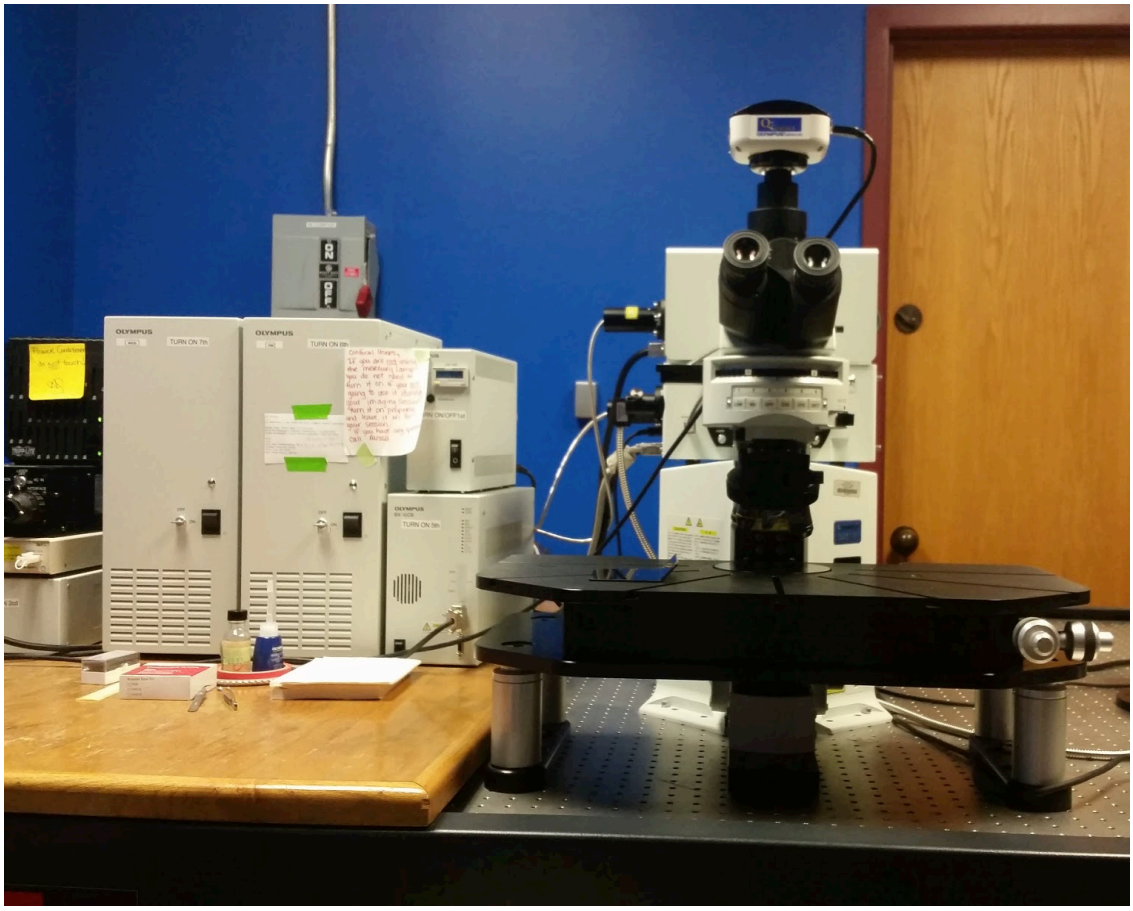


Figure 6A: Olympus FV1000 confocal laser-scanning microscope used for image acquisition.

Table 1: Summary of quantified data for GFAP expression. "-" indicates zebrafish sample from 3-month age group that did not provide usable optic nerve sections. Sample sizes of zebrafish used for each age group are as follows: 3 month (n=3), 6 month (n=4), 9 month (n=4) and 12 month (n=4). Units are pixel intensity per unit area.

Age_Fish#_Sex_Series#_	**Nuclei Count**	**Average GFAP Integrated Density**	**Average Integrated Density/Nuclei**
3Mo_Fish1_Male_Series1_	30	27289	909.63
3Mo_Fish2_Female_Series1_	18	18214	1011.89
3Mo_Fish3_Male_Series2_	24	0	0
3Mo_Fish4*_Female_Series3	-	-	-
6Mo_Fish1_Male_Series1_	33	17714	536.79
6Mo_Fish2_Female_Series1_	45	24535	545.22
6Mo_Fish3_Male_Series2_	59	909164	15409.56
6Mo_Fish4_Female_Series3_	22	544629	24755.86
9Mo_Fish1_Female_Series1_	51	96389	1889.98
9Mo_Fish2_Male_Series1_	29	287227	9904.38
9Mo_Fish3_Male_Series2_	64	198622	3103.47
9Mo_Fish4_Female_Series3_	105	543702	5178.11
12Mo_Fish1_Male_Series1_	97	1537910	15854.74
12Mo_Fish2_Female_Series1_	248	2819103	11367.35
12Mo_Fish3_Female_Series2_	61	2599441	42613.79
12Mo_Fish4_Male_Series3_	84	824824	9819.33

Table 2: Summary of quantified data for p16-ARC expression. "*" indicates zebrafish sample from 3-month age group that did not provide usable optic nerve sections. "**" indicates zebrafish sample in which the optic nerve sections did not suffice to conduct p16-ARC experiment. Sample sizes of zebrafish used for each age group are as follows: 3 month (n=3), 6 month (n=4), 9 month (n=4) and 12 month (n=4). Units are pixel intensity per unit area.

Age_Fish#_Sex_Series#_	**Average p16-ARC Integrated Density**
3Mo_Fish1_Male_Series1_	**
3Mo_Fish2_Female_Series1_	24278
3Mo_Fish3_Male_Series2_	11782
3Mo_Fish4*_Female_Series3_	-
6Mo_Fish1_Male_Series1_	63013
6Mo_Fish2_Female_Series1_	51471
6Mo_Fish3_Male_Series2_	49002
6Mo_Fish4_Female_Series3_	32491
9Mo_Fish1_Female_Series1_	183521
9Mo_Fish2_Male_Series1_	219511
9Mo_Fish3_Male_Series2_	146313
9Mo_Fish4_Female_Series3_	946313
12Mo_Fish1_Male_Series1_	621433
12Mo_Fish2_Female_Series1_	492914
12Mo_Fish3_Female_Series2_	952259
12Mo_Fish4_Male_Series3_	775360

Table 3: Summary of quantified data for p16-ARC expression. * Indicates zebrafish sample from 3-month age group that did not provide usable optic nerve sections. Sample sizes of zebrafish used for each age group are as follows: 3 month (n=3), 6 month (n=4), 9 month (n=4) and 12 month (n=4).

Age_Fish#_Sex_Series#_	**Optic Nerve Diameter ($\mu m^{2)}$**
3Mo_Fish1_Male_Series1_	110
3Mo_Fish2_Female_Series1_	89.4
3Mo_Fish3_Male_Series2_	117
3Mo_Fish4*_Female_Series3_	-
6Mo_Fish1_Male_Series1_	183.3
6Mo_Fish2_Female_Series1_	190
6Mo_Fish3_Male_Series2_	186.7
6Mo_Fish4_Female_Series3_	215
9Mo_Fish1_Female_Series1_	211.7
9Mo_Fish2_Male_Series1_	210
9Mo_Fish3_Male_Series2_	226.7
9Mo_Fish4_Female_Series3_	198.9
12Mo_Fish1_Male_Series1_	250
12Mo_Fish2_Female_Series1_	260
12Mo_Fish3_Female_Series2_	268.9
12Mo_Fish4_Male_Series3_	266.5

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