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Optimization of tyrosine hydroxylase antibody for immunohistochemistry fluorescence detection in zebrafish (Danio rerio)

Madison Thurber

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Abstract

Dopamine is an important neurotransmitter produced through the catecholamine synthesis pathway that affects brain activity. Unregulated dopamine levels can lead to various diseases such as Parkinson's Disease or attention deficit hyperactivity disorder (ADHD). Optimization of an immunohistochemistry protocol will allow for the quantification of tyrosine hydroxylase antibody, which indirectly allows for dopamine quantification in dopaminergic regions within the brain. However, the antibody concentration to give the optimal signal-to-noise ratio in IHC varies across studies. Through this experiment, I determined the concentration of tyrosine hydroxylase (TyrH) antibody for immunohistochemistry that gave the best signal-to-background noise ratio within several known dopaminergic regions in the zebrafish brain. I tested the following primary antibody concentrations: 1:200, 1:500, 1:750, and 1:1000. While there was a significant difference in signal-to-noise ratio amongst the concentrations as determined by a Friedman's ANOVA, the Wilcoxon Test failed to yield a significant difference between treatment groups. These findings allow for future experiments on the correlations between neurotransmitters and personality types in zebrafish.

Introduction

Dopamine is one of several catecholamine neurotransmitters (norepinephrine, dopamine, and epinephrine). Dopamine is involved with neurological processes such as movement, memory, reward, motivation, behavior, cognition, and attention (Ayano, 2016). While dopaminergic neurons make up less than 1% of neurons, their effect is profound on brain activity (Arias-Carrion & Poppel, 2007). Unregulated levels of dopamine can have detrimental health effects such as Parkinson's Disease, attention-deficit hyperactivity disorder (ADHD), and major depressive disorder (Franco, et al., 2021). Many therapies for these diseases target intermediates in the catecholamine synthesis pathway (Nagatsu, 2007).

The catecholamine synthesis pathway is responsible for producing dopamine as well as the two other catecholamines: norepinephrine and epinephrine (Daubner & Wang, 2010). Tyrosine hydroxylase (TyrH) is the rate-limiting enzyme within the catecholamine synthesis pathway. The pathway begins when TyrH catalyzes the addition of a hydroxyl group into the meta position of L-tyrosine which then becomes L-DOPA. DOPA decarboxylase catalyzes the removal of the carboxyl group that converts L-DOPA into dopamine (Kuhar, et al., 1999). Dopamine can then either be packaged into synaptic vesicles for use as a neurotransmitter or be converted into norepinephrine or epinephrine through additional chemical reactions.

While TyrH was only discovered in 1964 by Natasgu (Nagatsu, 1964), it has been used in numerous studies involving dopamine production in mice. One study found that a mutant phenotype prohibiting the production of dopamine was fatal until the introduction of the TyrH enzyme corrected the phenotype into producing dopamine (Zhou, et al., 1995). Additionally, a study mutated the TyrH enzyme to produce 40% of the typical amount, which caused significant impairment physically and neurologically in terms of memory and fear conditioning (Kobayashi, et al., 2000). These studies combine to demonstrate the importance of dopamine produced via the catecholamine synthesis pathway for normative functions of the organism.

Immunohistochemistry (IHC) can be performed to indirectly determine the presence of neurotransmitters such as dopamine. In general, IHC is a histological process that uses antibodies to detect proteins within a tissue sample. IHC involves a primary antibody solution, a secondary antibody solution, and microscopy. The primary antibody solution is prepared with a blocking solution to prevent the primary antibody and later the secondary antibody from binding to anything but the intended target. Noise occurs when a secondary antibody binds to structures other than the desired primary antibody. Optimization of protocols can help alleviate noise by

manipulating aspects of the IHC protocol to allow for more accurate binding of the applied antibodies. The optimized protocols may differ for each laboratory, which is why it is important to identify parameters before using the protocols. As demonstrated in Figure 1 obtained from Salameh, et al., 2017, protocol optimization can dramatically enhance microscopic images. A lack of optimization in IHC protocols can generate inaccurate quantification of the protein visualized through microscopes. When using IHC to analyze dopamine, a lack of optimization can inaccurately represent areas of dopamine localization within the brain. This can lead to incorrect assumptions and understanding of dopaminergic regions in the brain (Salameh, et al. 2017).

In my experiment, I used various concentrations of primary antibody in order to optimize the binding of secondary antibodies to the primary antibody. The primary antibody is manipulated because it is applied with the blocking solution. This means that when the primary antibody is manipulated in concentration, it is able to bind to more intended targets. Since the secondary antibody is applied after the blocking solution, the secondary antibody is only binding to the primary antibody and visually reflects the areas that the primary antibody has previously bound. Thus, the noise can be generated from the primary antibody binding to regions outside of the intended target, which is visualized with the secondary antibody. In this experiment, I used four different concentrations of primary antibody (anti-TyrH) within an IHC protocol to determine the optimal signal-to-noise ratio. I hypothesized that a 1:500 concentration will produce the optimal signal-to-noise ratio as it is the median value given on the manufacturer's website of suggested concentrations (Sigma-Aldrich, Darmstadt, Germany).

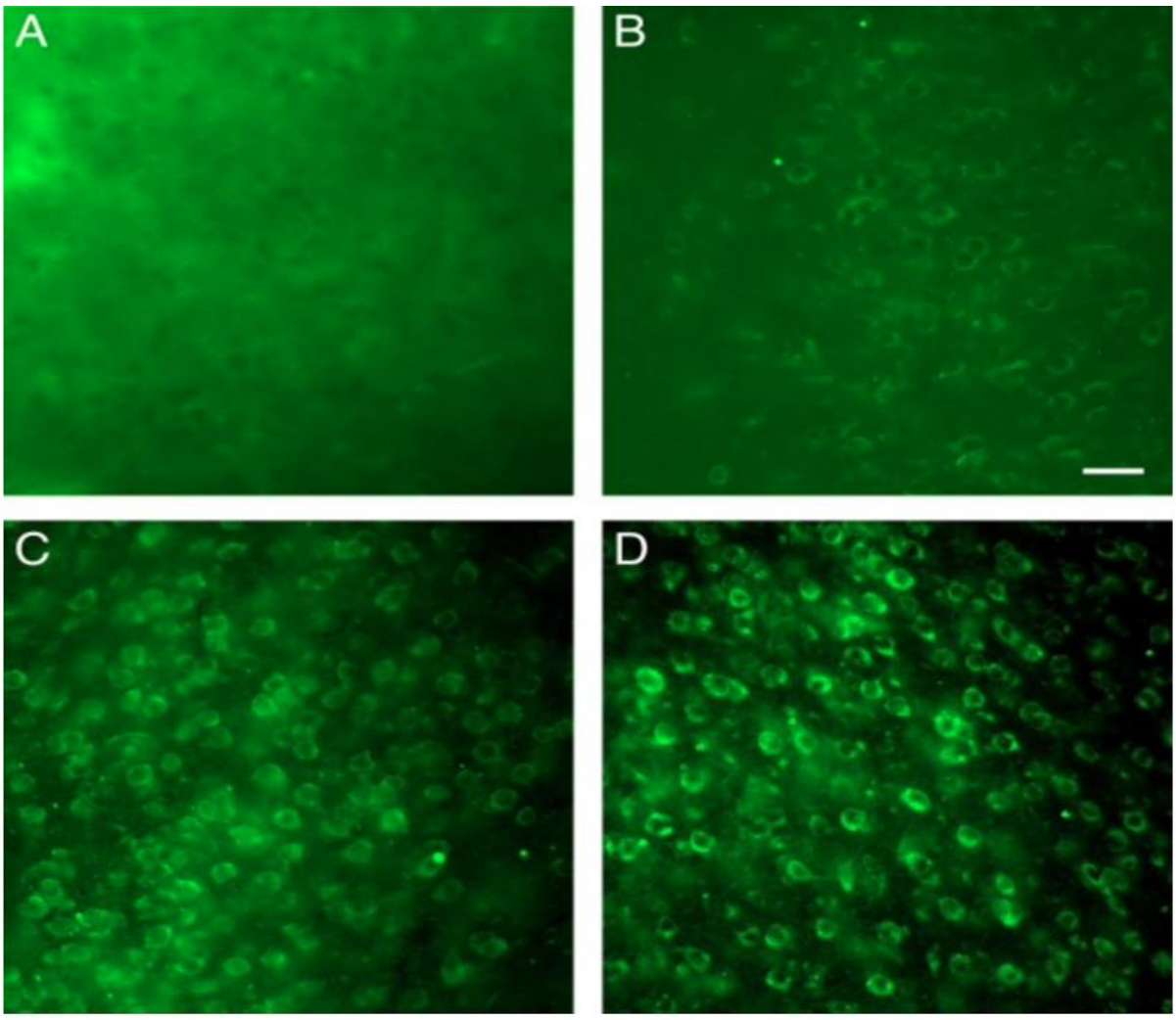

Figure 1: Optimized TyrH images obtained from Salameh, et al., 2017. Image A is the least optimized, and Image D is the most optimized.

Methods/Materials:

In this experiment, I used IHC to detect the presence of tyrosine hydroxylase inside the zebrafish brain. I fixed collected brain tissue to allow the sections to become more suitable to antibody binding before IHC can take place. Fixation is the process of immobilizing the antigens on the surface of a section while preserving all cellular structures. This is typically done with paraformaldehyde and ethanol washes (Abcam). IHC is performed in a sequence of steps: addition of primary antibody, secondary antibody, and microscopy (Magaki, et al., 2019). Anti-

TyrH is used as the primary antibody because tyrosine hydroxylase is the precursor to dopamine, and it is present at all steps in the pathway (Hamanaka, et al., 2016). Sigma-Aldrich, the antibody manufacturer, suggests the recommended concentration range be from 1:200-1:1000. The lower concentration numbers, 1:200, have a higher anti-TyrH to solution ratio than the higher concentration numbers, 1:1000. I assessed the signal-to-noise ratio within select zebrafish brain regions for four different concentrations of anti-TyrH antibody. For this experiment I prepared each zebrafish head (N=4) using the same fixation protocol and the same IHC protocol.

Test Species

Zebrafish, *Danio rerio*, were housed in 40L fish tanks and fed twice a day with Tetramin Tropical Flakes (Tetra, USA) on a recirculating water system (Pentair Aquatic Ecosystems) using UV and solid filtration on a 14:10 L/D cycle at a temperature of 27°C. All procedures in my experiment were approved by UNO IACUC (17-070-09-FC). Other individuals within the lab performed euthanasia of zebrafish in the months prior to this experiment.

I stored the heads at -80°C until the time of cryosection. Before cryosectioning, I engulfed the heads in freezing medium (Fischer Scientific, USA) and allowed them to fully freeze. I serially sectioned the heads onto four series with 16-micrometer-thick sections. These sections were then stored in the -80°C freezer until fixed.

Fixation

I dipped the slides into ice-cold 4% PFA/PBS for 10 minutes followed by a one-minute PBS wash. Then, I dipped the slides into Triethanolamine (TEA) solution (pH of 8.0, 10 minutes). I washed the slides in TEA solution with 0.25% acetic anhydride for 15 minutes. Next, I washed the slides in 2x SSC twice for one minute each time. Then, I dipped the slides into 30-100% ethanol (2 minutes). After dipping, I allowed the slides to dry for 10-40 minutes and placed into the -80°C freezer.

Immunohistochemistry

I tested four concentrations of primary TyrH antibody (one per series; SAB2701683, Sigma-Aldrich): 1:200, 1:500, 1:750, and 1:1000. I selected these varying concentrations due to conversations with lab faculty and additional information obtained via the antibody supplier (Sigma-Aldrich, Darmstadt, Germany). This TyrH antibody is an affinity-isolated, polyclonal, unconjugated, buffered aqueous solution antibody produced in rabbits. I defrosted the slides for 10-20 minutes until fully dry and used ImmEdge™ PAP Pen (Vector Laboratories, USA) on the edges of the slide. Next, I washed the slides twice in 1x PBS for 5 minutes. I washed the slides in citric acid solution (pH of 6.0, 2-5 minutes, 82.5°C). I washed the slides in 1X PBS twice for five minutes each time. While the slides were washed, I added 1x PBS to the bottom of a dark box. I placed the slides into the dark box where I then added a blocking solution to the top of each slide (Table 1). I left the slides in the box at room temperature to incubate for 60 minutes, removed the blocking solution, and added primary antibody solution (Table 2) to the top of the slides. I transferred the dark box into the 4°C refrigerator and incubated for 48 hours.

Blocking Solution	Percentage	Volume
Serum	5%	270 µL
Triton X-100	0.3%	16.2 µL
1X PBS	To Volume	5.1138 mL

Table 1: Calculations based on 300 µL on each of the 18 slides. Serum is Normal Goat Serum.

Primary Antibody	Percentage	Volume
Anti-TH	1:200, 1:500, 1:750 or 1:1000	27, 10.8, 7.2, 5.4 µL
Serum	2%	108 µL
Triton X-100	0.3%	16.2 µL
1x PBS	To Volume	5.248 mL

Table 2: Calculations based on 300 µL on each of the 18 slides. Serum is Normal Goat Serum. Anti-th is antibody produced in rabbit from Sigma-Aldrich

Secondary Antibody	Percentage	Volume
Fluorescence	1:200	27 µL
Serum	2%	108 µL
Triton X-100	0.3%	16.2 µL
1x PBS	To Volume	5.248 mL

Table 3: Calculations based on 300 µL on each of the 18 slides. Serum is Normal Goat Serum.

I washed the slides in PBS for 10 minutes (2 times). All the following steps were light-sensitive and were performed in a dark environment. I placed the slides into the dark box and added a secondary antibody solution (Table 3) to the slides (Alexa Fluor 488 goat anti-rabbit, A-11008, Thermo Fisher Scientific, USA). I incubated the slides for 90 minutes and washed the slides twice in 1x PBS for 10 minutes. I washed the slides in DAPI/PBS solution for four minutes at room temperature. Next, I washed the slides in 1x PBS three times for 5 seconds each. I applied 5 drops of Prolong Diamond to each slide and placed the coverslip. I left the slides overnight in the dark box until the Prolong Diamond had completely dried. Once the slides were dry, I placed them into a -20°C freezer for storage. After the protocol was completed for all four concentrations of primary antibody, I analyzed the slides under a microscope.

Visualization

I visualized slides using a Nikon Eclipse microscope (Nikon, USA), and the Nikon DS-Qi2 monochrome microscope camera (Nikon, USA) within the NIS-Elements Br software program (Nikon, USA). I visualized the slides using a 10x objective. I adjusted the image intensity to be 16383K for each slide. I used a FITC filter to capture images of the TyrH antibody and a DAPI filter to observe cells to confirm the presence of cells. I examined each concentration's slides for fluorescent signal in the olfactory bulb, the dorsal nucleus of the ventral telencephalic area (Vd), and the suprachiasmatic nucleus (SC) (Kaslin & Panula, 2001). These regions are known for containing dopaminergic neurons, which in turn have high tyrosine hydroxylase production (Kaslin & Panula, 2001).

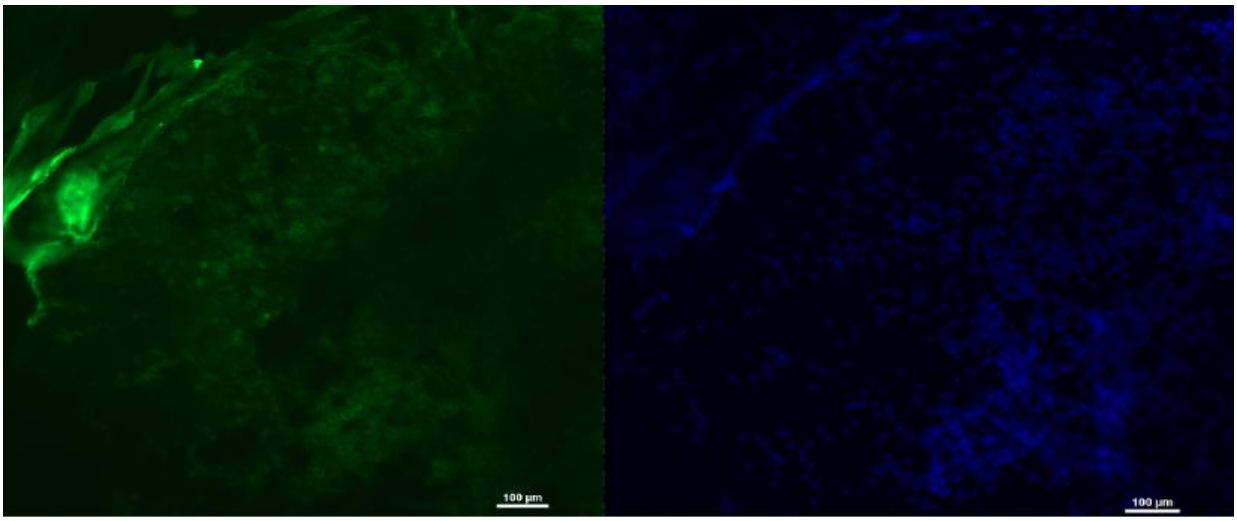

Figure 2: Zebrafish 1 treated with 1:1000 concentration visualizing the olfactory bulb. The left image is visualized using a FITC filter. The right image is the DAPI stain. Bright green staining on the left side of the FITC image is assumed to not be signal, but bright staining of bone or dense fixture in the slide. The entire image is the olfactory bulb.

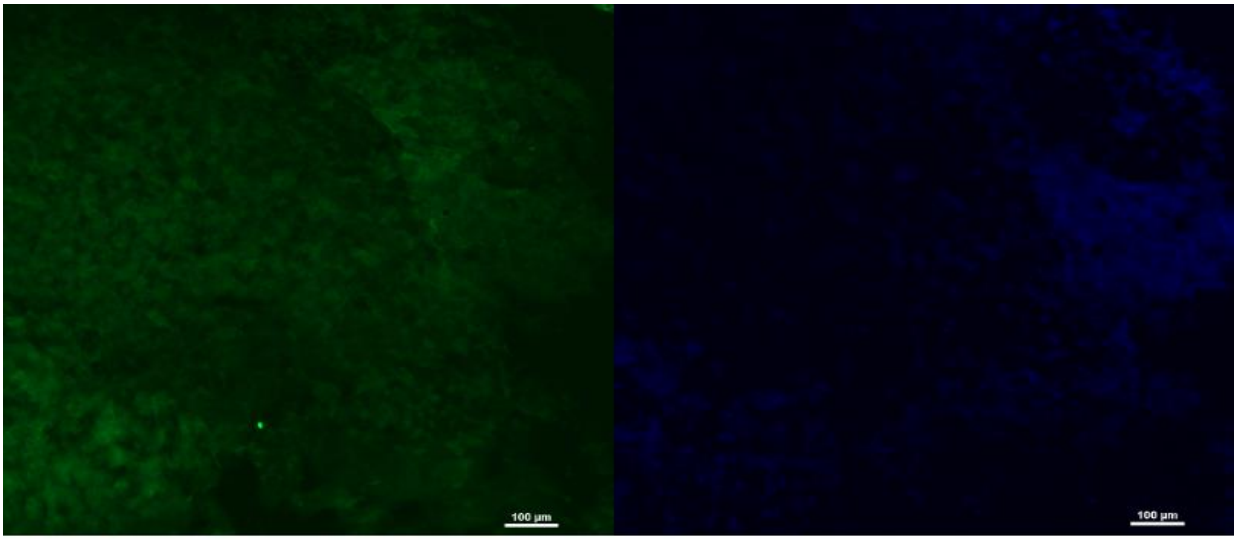

Figure 3: Zebrafish 1 treated with 1:1000 concentration visualizing the SC. The left image is visualized using a FITC filter. The right image is the DAPI stain. The entire image is the SC.

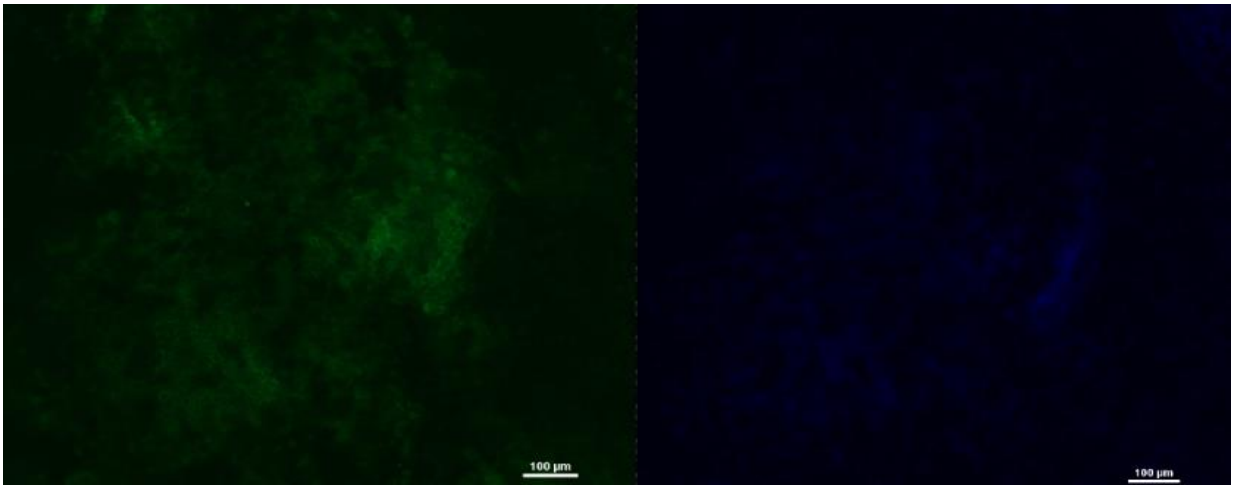

Figure 4: Zebrafish 1 treated with 1:1000 concentration visualizing the VD. The left image is visualized using a FITC filter. The right image is the DAPI stain. The entire image is the VD.

Analysis

I obtained regions of interest (ROIs) from the TyrH images containing a background ROI and a signal ROI (Figure 5). One signal ROI and one background ROI were captured per image. The signal ROI was placed on the brightest area of binding to the brain (not the skull as it produced a more intense fluorescent product), and the background ROI was placed in an area that had little-to-no signal. This ensured that the areas captured were indeed both locations of signal and locations of noise. The ROI's size varied to receive the most accurate data (1,000-3,000 micrometers^2). The signal ROI was very small to ensure that the signal was the only fluorescence captured. The background ROI was larger than the signal ROI, but it was small enough to avoid capturing the signal in the ROI (25,000 to 50,000 micrometers^2). The data obtained from the ROI included minimum intensity, mean intensity, maximum intensity, standard deviation intensity, sum intensity, and signal/background intensity.

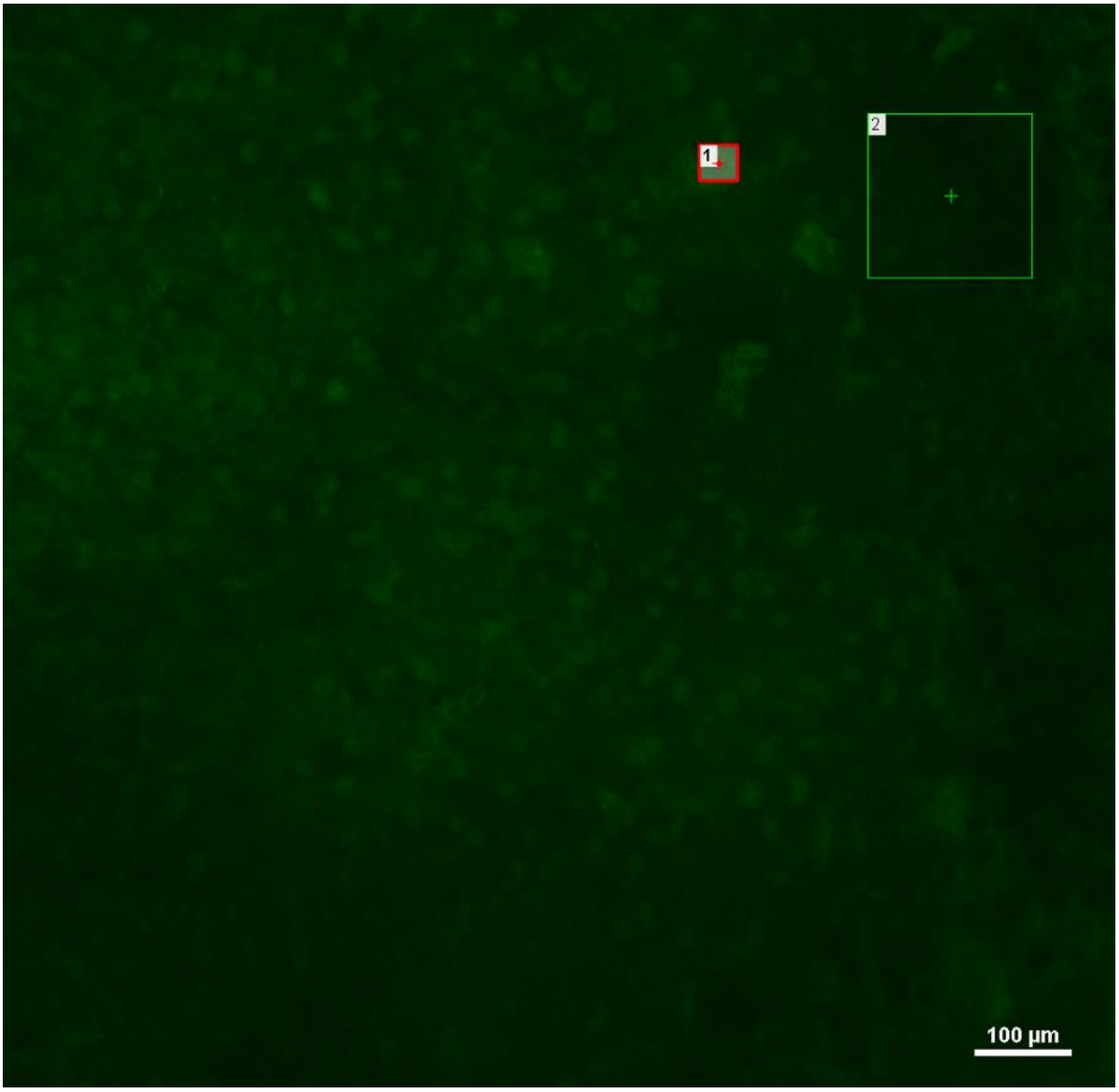

Figure 5: Box 1 (red) is a signal ROI that is small to ensure that signal was the only fluorescent intensity captured. Box 2 (green) is a background ROI that is large enough to capture a large amount of noise within the background. This is within the left olfactory lobe of an unknown zebrafish strain treated within the 1:1000 treatment group.

I converted mean intensity to optical density, OD, using the formula: 2-log(mean intensity). This was done for both the signal and the background intensity. I found the optical density ratio of signal to background using the optical density of the signal divided by the optical density of the background. I repeated this process for each image obtained (three images per

brain region per fish) and sorted the data into groups by concentration. Some images were unable to be used due to the folding or tearing of the brain during cryosectioning. I used Friedman's repeated measures one-way ANOVA to investigate the effect of the primary antibody concentrations on the optical density ratios. For this analysis, the OD ratio was averaged into one value per fish to ensure that each treatment group would have the same sample size. Following this ANOVA, I used a Wilcoxon Test to determine the statistical significance of the sets of pairs of concentrations. To look for differences in OD ratios between brain regions within a concentration, I used a Wilcoxon test.

Results:

There was a significant main effect of the primary antibody concentration on the optical density ratio (N=4, p=.017 Q=10.2, df=3, W=0.85, Fig 6). The Wilcox Test determined that none of the concentrations are statistically significant in comparison to each other (Table 4). Despite the differences in OD ratio between groups not being significant, the highest signal-to-noise optical density ratio was found in the 1:1000 treatment group. Figure 7 shows the images obtained from all four concentrations within the same brain region. The 1 to 200 concentration demonstrated a high intensity of staining for all regions indicated by a heavy presence of green within the background ROI and the signal ROI (Fig 7A). The 1 to 500 concentration demonstrated a very limited amount of staining for all regions which is indicated by little presence of green within the background ROI and signal ROI (Fig 7B). Conversely, the 1 to 750 concentration demonstrated an increase in staining of both background ROI and signal ROI (Fig 7C). The 1 to 100 concentration shows an increase in accurate staining of the signal ROI inside the individual cells and limited fluorescence outside the cell in the background ROI (Fig 7D).

None of the brain region's optical density ratios are statistically significant in comparison to each other (Table 5-8).

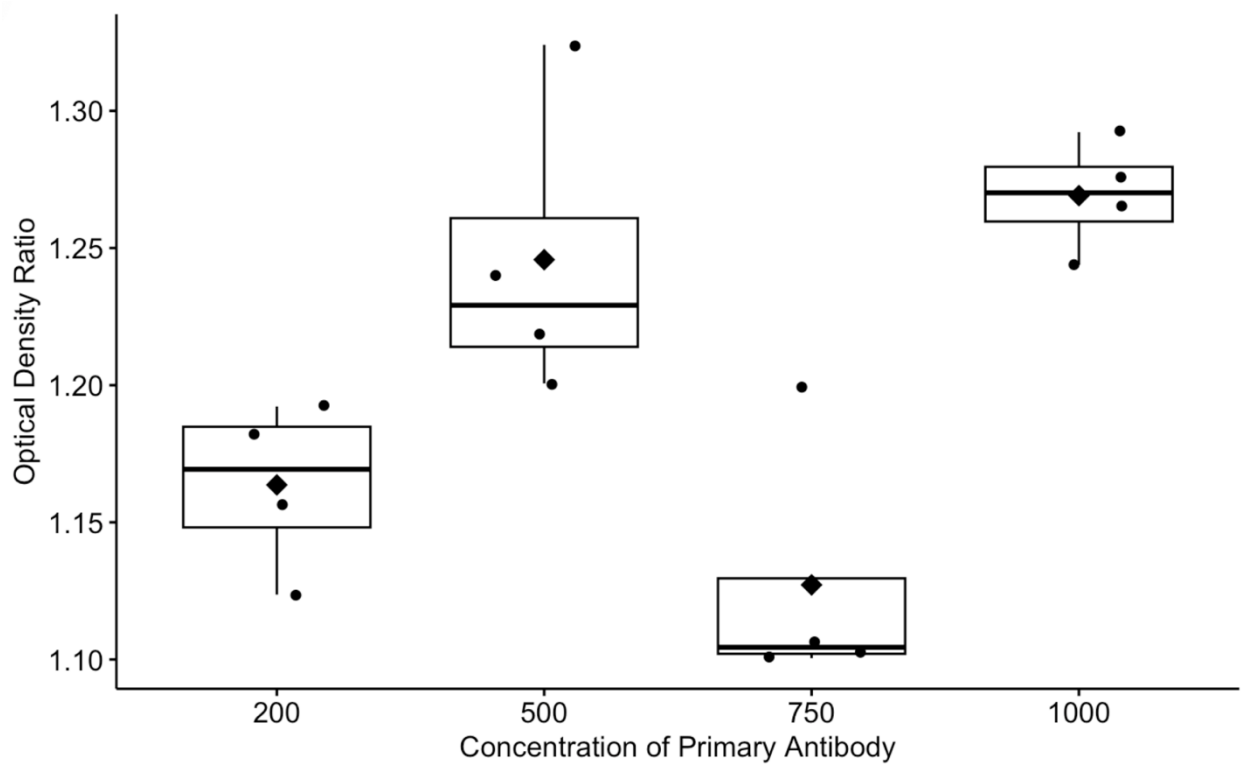

Figure 6: Friedman's multiple measures one-way ANOVA data represented in a boxplot. The diamond is the mean OD ratio for the concentration, and the horizontal line inside the box is the median OD ratio. The circles represent each fish's average OD ratio.

Concentration 1	Concentration 2	W	*p-value*	*p* adjusted
1 to 200	1 to 200	0	0.125	0.75
1 to 200	1 to 750	9	0.25	1
1 to 200	1 to 1000	0	0.125	0.75
1 to 500	1 to 750	10	0.125	0.75
1 to 500	1 to 1000	2	0.375	1
1 to 750	1 to 1000	0	0.125	0.75

Table 4: Wilcoxon Test data, corrected for multiple comparisons using the Bonferroni method.

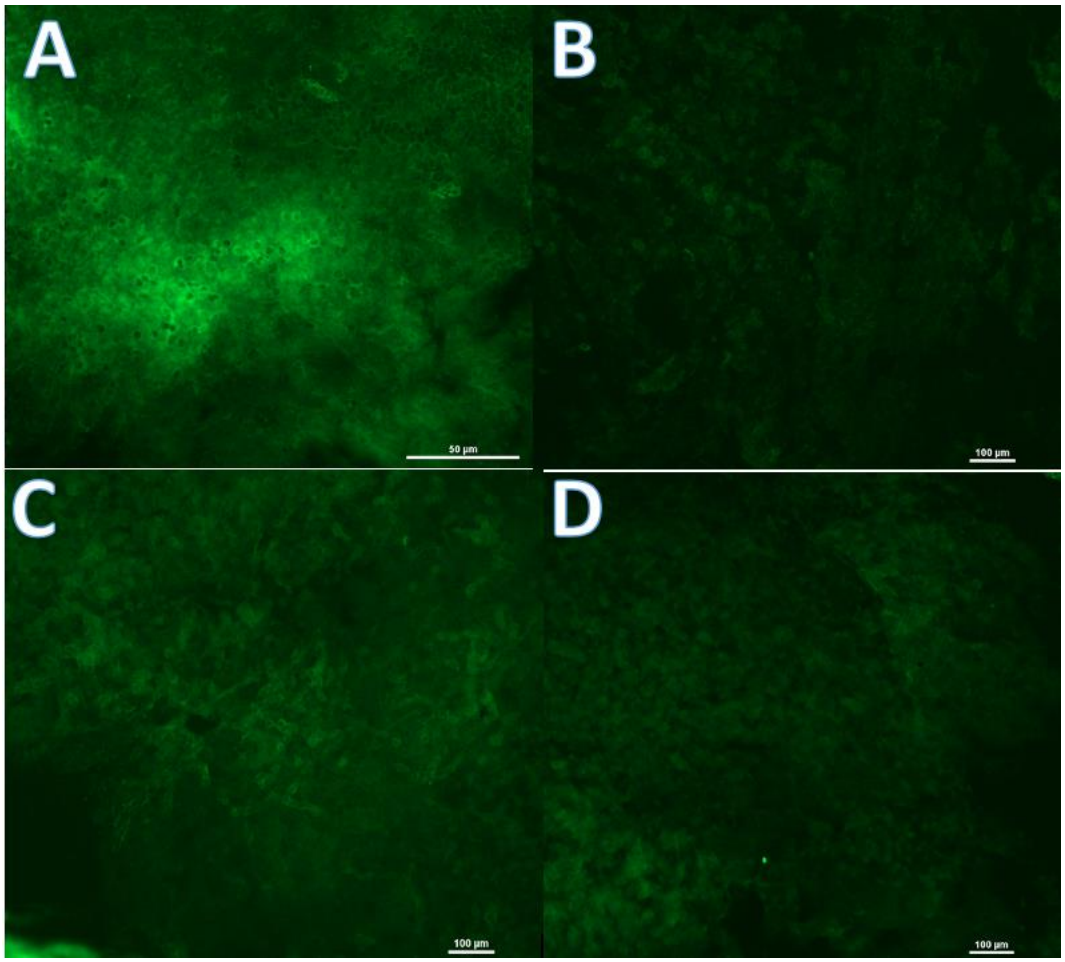

Figure 7: Zebrafish 2 in decreasing primary antibody solution concentrations visualizing the SC. A is 1:200, B is 1:500, C is 1:750, and D is 1:1000.

	OB	*SC*
SC	*0.50*	
VD	*0.64*	*0.21*

Table 5: Wilcoxon Test p-values on 1:200 concentration , corrected for multiple comparisons using the Bonferroni method

	OB	SC
SC	0.8	
VD	0.8	0.8

Table 6: Wilcoxon Test p-values on 1:500 concentration , corrected for multiple comparisons using the Bonferroni method

	OB	SC
SC	0.82	
VD	0.82	0.82

Table 7: Wilcoxon Test p-values on 1:750 concentration , corrected for multiple comparisons using the Bonferroni method

	OB	SC
SC	1	
VD	1	1

Table 8: Wilcoxon Test p-values on 1:1000 concentration , corrected for multiple comparisons using the Bonferroni method

Discussion:

The predicted optimized concentration was 1:500. The two highest OD ratios were the 1:500 and 1:1000 concentrations. The decreasing primary antibody solution results in an increasingly high signal-to-noise ratio. I hypothesize that a lower concentration than 1:1000 will follow this trend, but more experiments are necessary to confirm. Friedman's one-way repeated measures ANOVA of the effect of the concentrations on the optical density ratios yielded a p-value of .017 (N=4, Q=10.2, df=3, W=0.85). This was a significant result indicating that at least the average optical density ratio of one of the concentrations was different from the others. However, a follow-up Wilcoxon test did not indicate any difference between the individual concentrations. From these analyses, it can be interpreted that the hypothesis is somewhat supported. The 1:1000 concentration had the highest median OD ratio which could indicate a necessity for a lower concentration of antibody to bind regions of the brain that were not in higher concentrations (Fig 6). However, the data represented a decrease in OD ratio in the 1:750 concentration compared to the 1:500 concentration. Without further experimentation with a larger concentration range, the cause of this is hypothesized to be a human error in performing the IHC protocol. The difference in OD ratio between the brain regions was not statistically different as determined by a Wilcoxon Test (N=4, df=3) (Table 5-8). The data fails to fully support my hypothesis or the data provided on the manufacturer's website. This failure to align with manufacturer suggestions demonstrates the importance of optimization of IHC variables within the lab where they are being used.

Many additional variables could be manipulated such as the method of fixation, secondary antibody concentration, and the blocking solution design. Optimization of TyrH IHC looks different for each laboratory. One lab focused on adding a pre-treatment trypsin solution resulted in increased TyrH binding and reduced background noise but used a concentration of 1:1000 TyrH antibody reared in rabbits (Towle, et al., 1983). In contrast, other studies in mice have manipulated the blocking solution to be applied for 48 hours followed by a TyrH concentration of 1:500 (Roostalu, et al., 2019). This data serves as a foundation for further experiments and as a general idea of the optimal concentrations for our IHC protocols.

The data of my experiment must be understood in the context of small sample size limitations. These numbers could have been skewed due to only analyzing three regions within four different zebrafish. While this experiment focused more on the concentrations and their effect on optical density, follow-up studies could investigate the effect of optimization on various brain regions and zebrafish strains. These experiments could alleviate this potential source of limitation. Additionally, these brain regions were solely located in the initial sections of the brain and did not span to the mid-brain. For example, the mid-brain contains the substantia nigra (SN) which is a dopaminergic nucleus whose dysregulation is linked to Parkinson's Disease (Sonne, et al., 2022). The SN is used to control movement (Sonne, et al., 2022) whereas the olfactory bulb is responsible for processing odor (Kaslin & Panula, 2001). These two functions are varied from each other and could possibly optimally bind at different concentrations. Although my data showed no significant differences in OD ratio between brain regions at any concentration, this may be due to low sample size. If further experimentation leads to varied binding optimization between brain regions, there could be a necessity to use separate protocols when performing IHC on the regions. Studying this region will allow for a further understanding of dopamine's role in

disease as well as an increased understanding of the role of brain regions in association with dopamine formation. These limiting factors could alter data in future trials with larger sample sizes and alternative brain regions.

Conclusion:

This experiment determined the optimal concentration of tyrosine hydroxylase within the primary antibody solution in fluorescent immunohistochemistry in *Danio rerio*. The 1:1000 concentration demonstrated the highest signal optical density to background optical density. However, this data is limited by a small sample size and analysis restricted to the forebrain. This experimentally derived data will be applicable to future experiments wanting to quantify similar neurotransmitters or use tyrosine hydroxylase as a reference point. This experiment serves as a starting point for the optimization of tyrosine hydroxylase fluorescent staining in the forebrain that can be applied to studies on the midbrain and hindbrain.

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