

**Bioassay of Vitamin A<sub>2</sub> in the Retinal Tissue of the zebrafish, *Danio rerio*, Using  
Microspectrophotometry and 2D-Imaging**

Honors Thesis

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## **ABSTRACT**

Zebrafish have been shown to utilize the vitamin A<sub>1</sub> chromophore in synthesizing their visual pigments. Recent evidence suggests that they may also be able to utilize vitamin A<sub>2</sub> under both natural conditions and after treatment with thyroxine. To test for the presence of vitamin A<sub>2</sub> in the retinae of zebrafish and map its location, I combined microspectrophotometric (MSP) analysis with 2D-imaging. MSP measures the absorbance spectra of visual pigments within single photoreceptors, while 2D-imaging uses differential bleaching to attempt differentiation of those regions rich in A<sub>1</sub> pigment from those containing A<sub>2</sub>. Previous MSP has found the occasional A<sub>2</sub> cell in some fish, but failed to demonstrate a specific area or island utilizing the A<sub>2</sub> chromophore in zebrafish, possibly due to sampling problems of the MSP technique. Unfortunately, the 2D-imaging failed to show enough contrast between bleached and unbleached retinal regions to determine if, in fact, bleaching occurred at all.

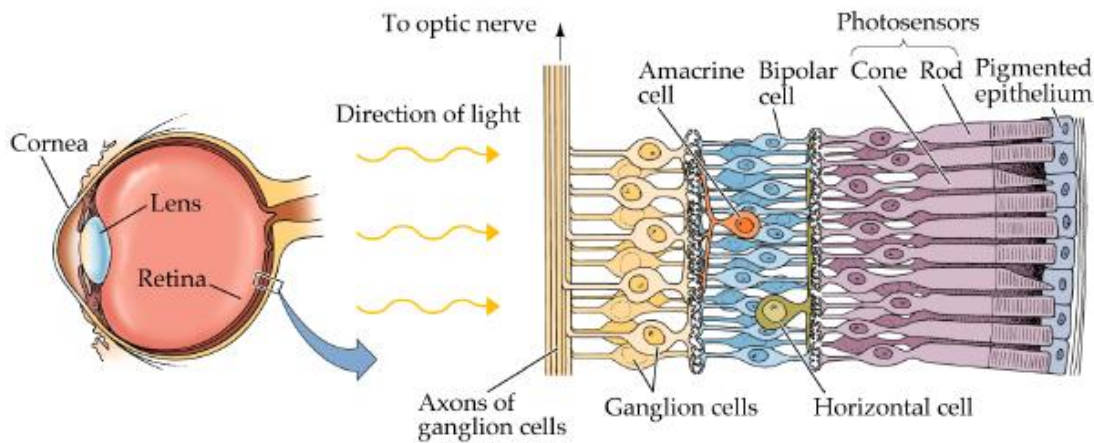
In an attempt to enrich the retina in vitamin A<sub>2</sub>, thyroxine treated eyecup cultures were used. In this case the hope was to stimulate A<sub>2</sub> synthesis in cultured eyecup preparations. MSP was used to monitor changes in vitamin A<sub>2</sub>-based pigment. There was a failure to observe A<sub>2</sub> synthesis in culture. While the most likely culture conditions were tried first, it is believed that these were NOT conducive to zebrafish retinal culture. This study has demonstrated methodological challenges that need to be solved before the original question can be satisfactorily addressed.

## **INTRODUCTION**

The eye is a sensory organ consisting of a lens that focuses light onto a layer of photoreceptors and a system of neurons that conduct action potentials from the receptors to the brain. Other principle components are the tough, outer casing called the sclera—the clear, anterior portion of which is called the cornea, a layer immediately inside the sclera called the choroid, and the innermost neural components, the retina and retinal pigment epithelium. Light rays enter the eye through the cornea and are focused by the lens onto the retina. The choroid is a layer rich with blood vessels that supply the outer retinal layers with nutrients. The layers of cells responsible for absorbing photons are the retina and the retinal pigment epithelium (RPE) (Ganong, 2005). Any light that is not absorbed by the retina is absorbed by the RPE. The RPE together with the choroid makes the globe of the eye light-tight except for the optic nerve entrance point and, of course, the pupil (Davson, 1990). This prevents photons from stimulating the photoreceptors from directions other than through the cornea, which would cause a decrease in contrast with a concomitant reduction in visual capability. The pigmented RPE lies between the retina and the choroid. (Ganong, 2005). This epithelial layer serves an important supportive role for the retina. The retina consists of rods and cones (photoreceptors), and four classes of neurons: bipolar cells, ganglion cells, horizontal cells, and amacrine cells (Davson, 1990; Ganong, 2005). Transduction of photon absorption into an electrical signal occurs in rods and cones, which then synapse to bipolar cells (Ganong, 2005). The bipolar cells synapse to ganglion cells whose axons course over the vitreal surface of the retina converging at the optic disk where they leave the eye forming the optic nerve (Davson, 1990). Horizontal cells allow for the transfer of electrical signals between

photoreceptors and carry signals laterally. They also participate in opponent and additive processing of color information. (Ganong, 2005). Lastly, the amacrine cells laterally connect ganglion cells via dendrites (Ganong, 2005) (Figure 1.1).

Figure 1.1. Diagram of the eye and close up of the retina. Light enters from the left and passes through the outer retina until it is absorbed by the rods and cones, with their outer segments embedded in the pigmented epithelium (retinal pigment epithelium).

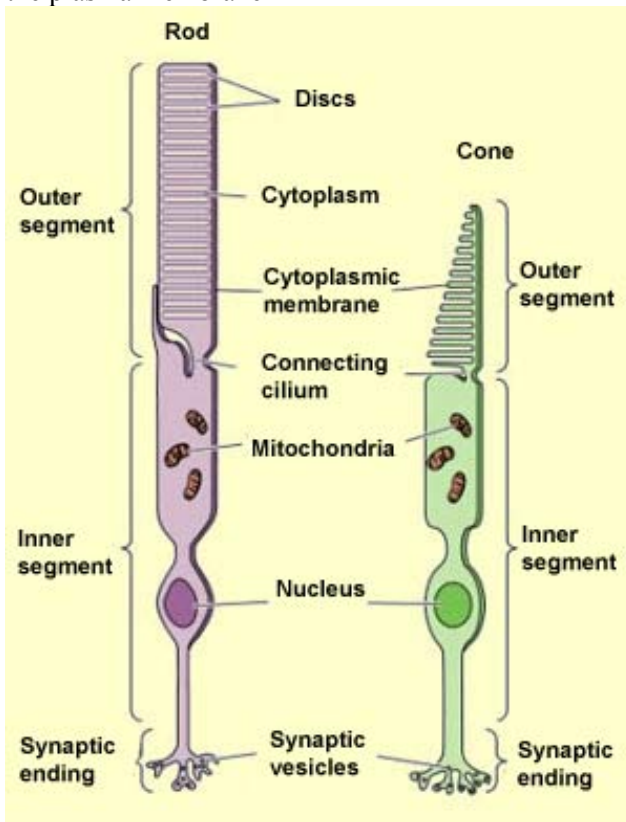


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Rods and cones are named after their morphology. These photoreceptors have inner and outer segments, a cell body, and a synaptic region (Alberts, Johnson, Lewis, Raff, Roberts & Walter, 2002). The outer segment is embedded in and surrounded by processes of the RPE and is the site of phototransduction (Davson, 1990; Burns & Lamb, 2004; Ganong, 2005; Alberts *et al.*, 2002). The inner segment is rich with mitochondria (Ganong, 2005), the cell body contains the nucleus (Alberts *et al.*, 2002), and the synaptic region is where the electrical signal synapses with a bipolar cell (Alberts *et al.*, 2002). The rod outer segment consists of a stack of individual membranous disks that are separated from the plasma membrane (Burns & Lamb, 2004; Ganong, 2005) (Figure 1.2).

Cones on the other hand consist of saccules that form from infoldings of the plasma membrane (Burns & Lamb, 2004; Ganong, 2005) (Figure 1.2). Embedded in the membranes of the disks and sacs are visual pigment molecules. It is light absorption by these pigments that endow photoreceptors with their sensitivity to light (Loew, 1995). All vertebrate visual pigments are composed of a protein, called opsin, and a covalently bound molecule of vitamin A (Ganong, 2005), called the chromophore.

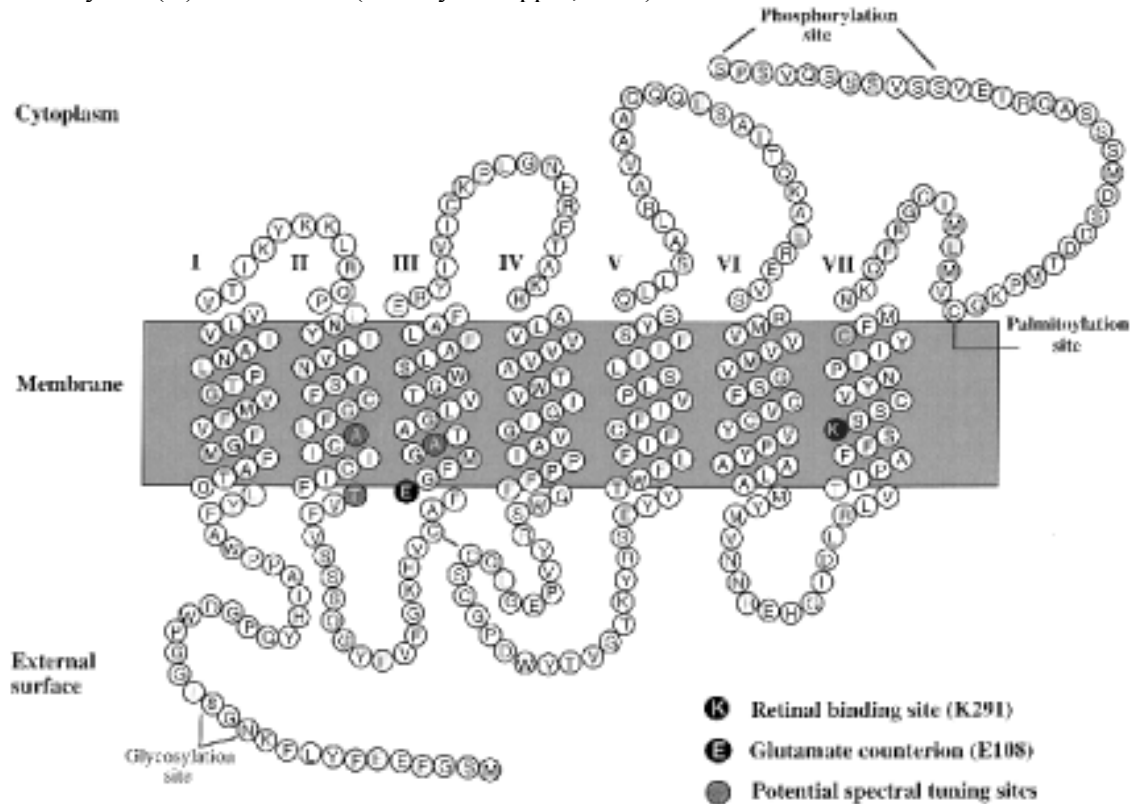
Figure 1.2. Diagram of rod and cone photoreceptors. The rod contains a stack of isolated disks separate from the plasma membrane, while the cone's saccules are formed from invaginations of the plasma membrane



An opsin is a protein that belongs to the superfamily of G-protein-linked receptors (Alberts *et al.*, 2002). These proteins are seven-pass transmembrane molecules utilizing a G-protein to transfer an extracellular stimulant to a second-messenger cascade in the interior of the cell (Alberts *et al.*, 2002). In the case of visual pigments, the extracellular

stimulant is a photon. Attached by a Schiff base linkage to a lysine occupying position 296 (on transmembrane helix VII) of the opsin is the chromophore (Figure 1.3) (Findlay, Pappin, 1986; Davson, 1990).

Figure 1.3. Opsin protein. Retinal is the aldehyde form of vitamin A (an alcohol). Retinal binds to the lysine (K) on helix VII (Findlay & Pappin, 1986).



Two forms of vitamin A are used by vertebrates in making a visual pigment. They differ in the number of alternating double bonds present. Vitamin A<sub>1</sub> (retinal) is the alcohol form of 11-*cis* retinal, which is an aldehyde. 11-*cis* retinal is one type of chromophore. Another is 3,4-didehydroretinal, which is vitamin A<sub>2</sub> in the alcohol form. Vitamins A<sub>1</sub> and A<sub>2</sub> are very similar in structure: the only difference is the presence of an additional double bond in the ring of vitamin A<sub>2</sub> (Figures 1.4 and 1.5). To date, there is no probe

that can distinguish between the two forms of vitamin A because of their structural similarities.

Figure 1.4. Chemical structure of vitamin A<sub>1</sub> (Ohtsu, Naito, Wilt, 1964)

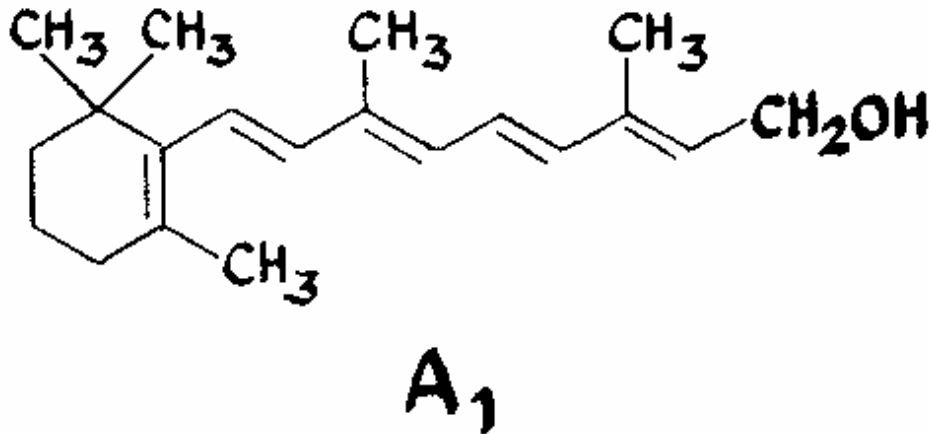
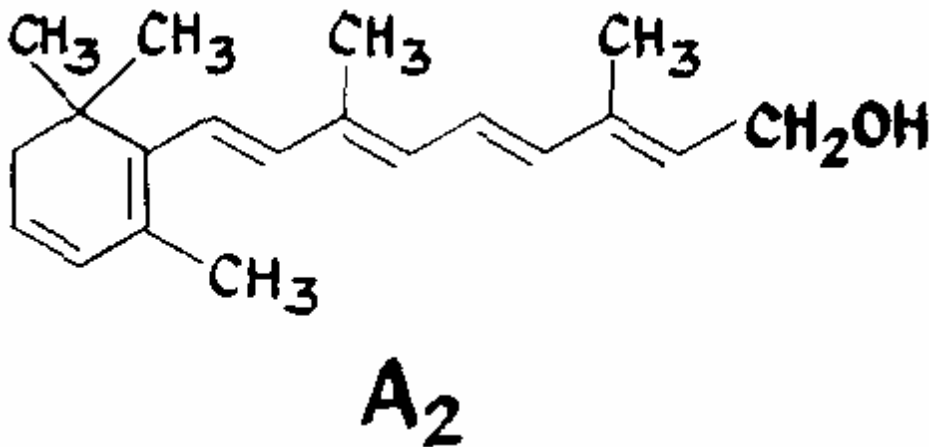


Figure 1.5. Chemical structure of Vitamin A<sub>2</sub> (Ohtsu, Naito, Wilt, 1964)



Regardless of whether 11-*cis* retinal or 3,4-didehydroretinal is used, both are bound to the opsin protein at the lysine on transmembrane helix VII. The absorption of a photon by the chromophore leads first to a conformational change in the chromophore: before absorption it is in the 11-*cis* configuration and after absorption it isomerizes to all-*trans* retinal (Davson, 1990; Burns & Lamb, 2004; Alberts *et al.*, 2002; Ganong, 2005). This

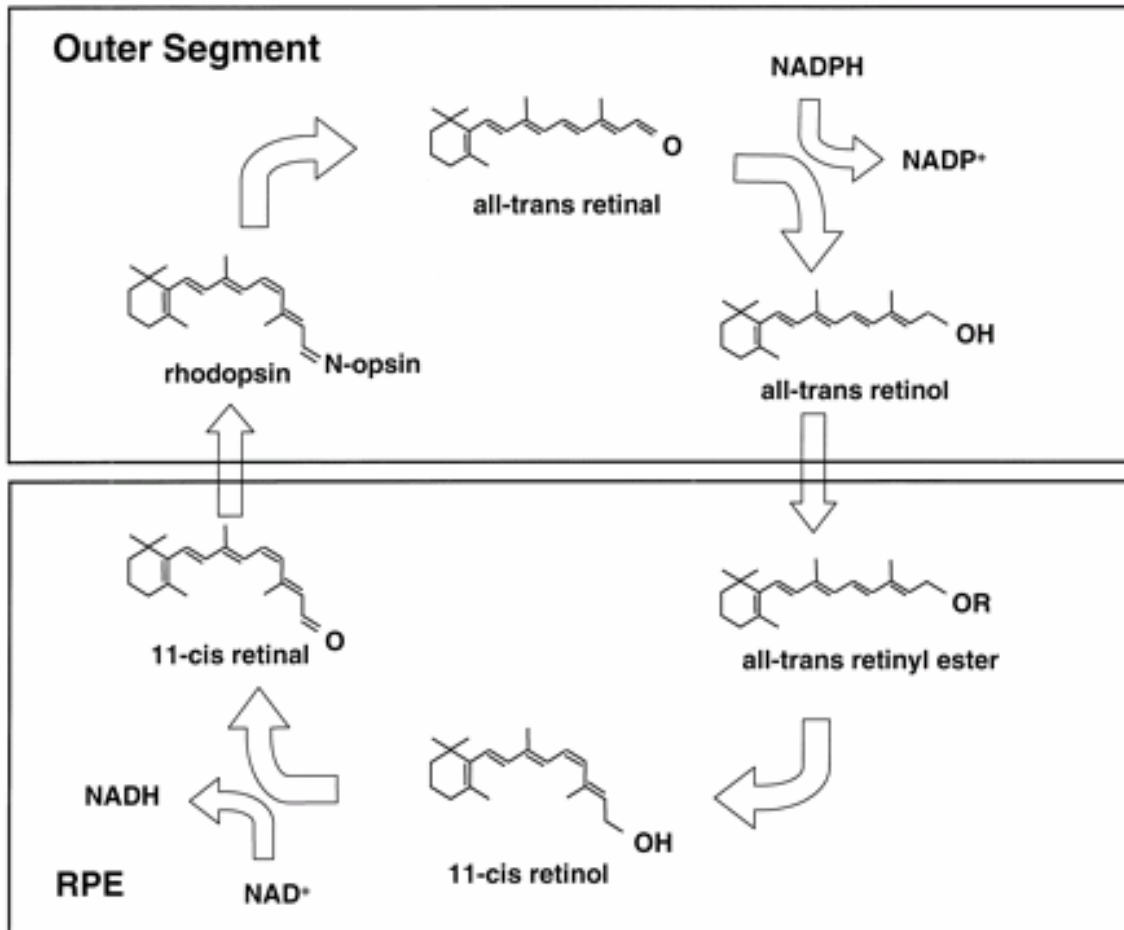
leads to a conformational change in the opsin that activates it, which in turn alters the G-protein *transducin* ( $G_t$ ) (Davson, 1990; Burns & Lamb, 2004; Alberts *et al.*, 2002). The  $\alpha$  subunit of the  $G_t$  dissociates and then activates cyclic GMP phosphodiesterase (Davson, 1990; Burns & Lamb, 2004; Alberts *et al.*, 2002). The cyclic GMP phosphodiesterase decreases the intracellular concentration of cyclic GMP via hydrolyzation (Davson, 1990; Burns & Lamb, 2004; Alberts *et al.*, 2002). Cyclic GMP is needed to keep  $Na^+$  channels in the plasma membrane of the photoreceptors open, so when the concentration falls, these channels close (Davson, 1990; Burns & Lamb, 2004; Alberts *et al.*, 2002). This ultimately leads to a change in membrane potential (Davson, 1990; Burns & Lamb, 2004; Alberts *et al.*, 2002) and the process is termed transduction. The only action of light is the *cis* to *trans* isomerization (Alberts *et al.*, 2002; Ganong, 2005). All other reactions can occur in the dark, such as the conversion from all-*trans* back to 11-*cis* by retinal isomerase (Figure 1.6) (Ganong, 2005).

The visual cycle represents the steps that transform 11-*cis* retinal to all-*trans* retinal and ultimately back to 11-*cis* retinal that can form functional visual pigment (Figure 1.6). Starting in the dark, the chromophore is in the 11-*cis* configuration, which sits in a hydrophobic pocket of the opsin (Davson, 1990; Burns & Lamb, 2004). When a photon is absorbed, the chromophore is provided with enough energy to isomerize to the all-*trans* configuration (Davson, 1990; Burns & Lamb, 2004). Changes in the conformation of the opsin reveal chemical groups that were formerly buried in the protein, such as SH-groups and  $H^+$ -binding sites (Davson, 1990). In order for the cycle to continue and the eye to remain sensitive to light, the products that result from photon absorption, or bleaching—all-*trans* retinal and opsin—must be converted back to



rhodopsin in the dark (Davson, 1990). The regeneration of these products in the dark—dark adaptation—depends on the relative amounts of all-*trans* retinal and all-*trans* retinol in the outer segment, which in turn is dependent on the proportions of NADP and NADPH (Davson, 1990). This cofactor is utilized by retinal dehydrogenase in the enzymatic conversion of retinal to retinol (Davson, 1990). The environment of the retina typically favors the production of retinol (Davson, 1990).

Figure 1.6. The visual cycle. The top box represents the reactions that occur in the rod outer segment, while the lower box is the RPE. When a photon is absorbed, 11-*cis* retinal in the RPE isomerizes to all-*trans* retinal in the outer segment. Retinal dehydrogenase utilizes NADPH to convert a product of bleaching, all-*trans* retinal to all-*trans* retinol, which is esterified in the RPE and converted back to vitamin A<sub>1</sub>, or 11-*cis* retinal (Ratter, Smallwood, Nathans, 2000).



The RPE is the source of chromophores for rods and cones (Loew, 1995). It is possible for both vitamin A<sub>1</sub> and A<sub>2</sub> chromophores to be used in one retina or even one photoreceptor; this ratio is established by enzymes in the RPE (Loew, 1995; Bridges, 1972). The presence of both forms of vitamin A in the zebrafish retina would change the way we understand the vision of this particular species. A specific dehydrogenase must produce vitamin A<sub>2</sub> from A<sub>1</sub>, but this enzyme has not been identified (Loew, 1995). The ratio of A<sub>1</sub>:A<sub>2</sub> is most likely maintained by this hypothesized dehydrogenase as well as the 3,4-hydrogenase which operates in the reverse direction (Loew, 1995). Naito and Wilt (1962) found direct evidence of the conversion of vitamin A<sub>1</sub> to 3,4-didehydroretinal in fish eyes. This conversion of vitamin A<sub>1</sub> to 3,4-didehydroretinal found by Naito and Wilt (1962) must occur in the visual cycle before its conversion to 11-*cis* retinal in the RPE.

Spectral sensitivity is determined by an animal's visual pigments, as well as any pre-retinal or intraphotoreceptor cell filters. A visual pigment is characterized by its absorption spectrum. Each visual pigment has a maximum sensitivity to light at a specific wavelength called the  $\lambda_{\max}$ . The visual pigment in rods is called rhodopsin if it utilizes vitamin A<sub>1</sub>, or porphyropsin if it utilizes A<sub>2</sub> (Burns & Lamb, 2004; Ganong, 2005). The opsin is in the RH1 family of opsins. In addition to the rod pigments, vertebrates have four classes of cones: a middle to long wavelength sensitive class (LWS) with  $\lambda_{\max}$  between 490-570 nm, a middle wavelength (RH2) class sensitive between 480-535 nm, a short wavelength sensitive class (SWS2) with  $\lambda_{\max}$  between 410-490 nm, and lastly an additional short wavelength class (SWS1) sensitive in the 355-440 nm region (see review of Loew & Bowmaker, in press). These spectrally distinct classes arose from

mutations in a duplication of the ancestral opsin gene (Loew & Bowmaker, in press). Peak sensitivity and spectral shape are determined by the opsin and the chromophore that it complexes with. There are several mechanisms available to an organism for shifting or ‘tuning’ its spectral sensitivity (Loew, 1995). These include: amino acid substitutions and post-translational modifications of opsins, and variation in the expression or activity of enzymes responsible for chromophore synthesis, among others (Loew, 1995). There are approximately eight sites in the opsin backbone where the amino acid sequence ultimately determines the absorbance spectrum (Bowmaker, 1991; Tovee, 1994; Loew, personal communication). Thus it is possible for a visual pigment’s  $\lambda_{\text{max}}$  to be shifted if there are amino acid substitutions affecting the conformation of opsin or if the chromophore is switched between vitamin A<sub>1</sub> and A<sub>2</sub>.

Many animals tune their spectral sensitivities over the course of their lives. This is beneficial because often an animal’s larval and adult environments will differ photically. The salmonids represent a family of fish that much is known about in terms of developmental and life history changes of the retina (Loew & Bowmaker, in press). Most developmental changes involve the gain and loss of ultraviolet (UV) photoreceptors (Loew & Bowmaker, in press). In Pacific salmonids it has been shown that there are only single UV sensitive cones present at hatching (Loew & Bowmaker, in press; Cheng, Novales Flamarique, Karosi, Rickers-Haunerland & Haunerland, 2006). This opsin is down-regulated later in development via apoptosis of the UV cone or increased expression of a gene producing a blue opsin (Loew & Bowmaker, in press). This shifts spectral sensitivity from the UV region to the blue. To improve visual fitness, spectral tuning is often necessary when the environments of the juvenile and adult are different.

In order for an animal to be the most visually fit, its visual system must be adapted to its environment. This is reflected in the contrasting visual systems of freshwater and marine fishes.

Freshwater environments are typically redder than their marine counterparts (Bridges, 1972; Loew, 1995). According to Bridges (1972) in deep oceanic water, only a small part of the photic spectrum exists. This light is centered in the blue-green region, with a  $\lambda_{\text{max}}$  at 480 nm (Bridges, 1972). Most fish living in these waters tend to have visual pigments with a  $\lambda_{\text{max}}$  in this spectral region (Bridges, 1972). Freshwater tends to be 'redder' due to the presence of plant pigments (gilvins) (Bridges, 1972). Interestingly, freshwater fish tend to have vitamin A<sub>2</sub> dominated visual systems while marine fishes utilize vitamin A<sub>1</sub> (Loew, 1995; Bridges, 1972; Wilt 1959). This is less surprising when we remember that the vitamin A<sub>2</sub> chromophore shifts absorbance to the red end of the spectrum when compared to its vitamin A<sub>1</sub> counterpart (Loew, 1995; Bridges, 1972). As previously stated, a visual system does not have to be entirely vitamin A<sub>1</sub> or A<sub>2</sub> based, there can be a mix (Bridges, 1972; Loew, 1995). Many species even utilize both vitamins A<sub>1</sub> and A<sub>2</sub> in a single photoreceptor (Loew, 1995). While the purpose of this type of spectral tuning has not been proven, it is the subject of much interest in the field of visual ecology.

Zebrafish are often used for research in vision and neural studies because so much is known about them at the molecular level. (Chinen, Hamaoka, Yamada, & Kawamura, 2003) They were long thought to possess a pure vitamin A<sub>1</sub> visual pigment complement (Allison, Haimberger, Hawryshyn, & Temple, 2004; Chinen *et al.*, 2003). But sometimes photoreceptors utilizing vitamin A<sub>2</sub> were found using microspectrophotometry (MSP)

although their number and location could not be confirmed due to the MSP technique, (Loew, personal communication). Furthermore, studies by Allison *et al.* (2004) and Chinen *et al.* (2003) have shown that vitamin A<sub>2</sub> synthesis can be induced with L-thyroxine. Studies do not show the presence and location(s) of these vitamin A<sub>2</sub> containing photoreceptors in the retinae of zebrafish.

Adult zebrafish have four morphologically distinct classes of photoreceptors (Loew & Bowmaker, in press; Robinson, Schmitt, Harositi, Reecet, & Dowling, 1993). There is one class of rod and three classes of cones: two single cones and a double cone (Figure 1.7) (Robinson *et al.* 1993). A double cone has a long, or principal member and a short, accessory member (Robinson *et al.* 1993). The principal member has a  $\lambda_{\text{max}}$  of 570 nm (when its opsin is bound to vitamin A<sub>1</sub>), which makes it red sensitive. This pigment is produced by the LWS gene as described by Loew and Bowmaker (in press). The accessory member is green sensitive and has a  $\lambda_{\text{max}}$  of 480 nm, corresponding to the RH2 gene (Robinson *et al.* 1993). One of the single cones is blue sensitive and has a  $\lambda_{\text{max}}$  of 415 nm (SWS2) and the other is UV sensitive, with a  $\lambda_{\text{max}}$  of 362 nm (SWS1) (Robinson *et al.* 1993).

Figure 1.7(a) Photograph of two single cones (vertical) and a rod (horizontal) in a zebrafish retina. (b) Photograph of a double cone in a zebrafish retina. The left member is the principal and the right member is the accessory.



(a)

(b)

Using microspectrophotometry (MSP) I measured the absorbance spectra of rods and cones in the retinas of zebrafish. My objectives were to look for the presence of vitamin A<sub>2</sub>-utilizing photoreceptors in zebrafish retinas, and to identify islands of these specific photoreceptors using MSP and 2D-imaging. As previously stated, the  $\lambda_{\max}$  of opsins using vitamins A<sub>1</sub> and A<sub>2</sub> differ, with those complexed with A<sub>2</sub> having a  $\lambda_{\max}$  shifted to about 750 nm (Loew, personal communication). MSP is the only technique

currently available for determining whether a photoreceptor is using A<sub>1</sub>, A<sub>2</sub> or both. This is because there is not enough difference between the two forms to allow for antibody production and virtually nothing is known about the pathways responsible for determining expression of one or both forms. While MSP is a useful tool for detecting the presence of vitamin A<sub>2</sub> utilizing cells, due to the nature of the technique, all spatial quality is lost. MSP can prove that these cells exist, but not where they are located in the retina. 2D-imaging exploits the different  $\lambda_{\max}$  but retains spatial information that is lost using MSP. According to Loew (personal communication) these cells may be located in islands, broadening the spectral sensitivity of zebrafish, thus changing our current understanding of how zebrafish see in nature. A combination of MSP and 2D-imaging represents an effective means of evaluating these aforementioned objectives because of the ability to detect and measure cells complexed with the vitamin A<sub>2</sub> chromophore and the ability to observe their location and relative quantity in the zebrafish retina.

As a test to see if MSP and 2D imaging could actually be utilized for the above purposes, and to determine their expected spatial resolution, eyes were treated with L-thyroxine in the hopes of increasing the number of cells utilizing A<sub>2</sub>. L-thyroxine was used to stimulate vitamin A<sub>2</sub> synthesis in a tissue culture using modifications of the work of Wilt (1959), Beatty (1969), and Chinen *et al.* (2003). The ultimate goal of all these studies was to provide information that could be incorporated into models of zebrafish visual ecology.

## **MATERIALS & METHODS**

### 2D-IMAGING

#### *Preparations Techniques*

Adult zebrafish were used in all experiments under an approved IACUC protocol. They were generously provided by the zebrafish holding facility (Mudd Hall, Cornell University, Ithaca, NY). The zebrafish were dark adapted and food was withheld for at least four hours before being anesthetized. All procedures followed were conducted under infrared illumination with suitable image converters or very dim red light. The fish were anesthetized using a 1% MS 222 solution (3-aminobenzoic acid ethyl ester). They were allowed to swim until they lost consciousness as evidenced by their inability to swim and eventually breathe. The animals had their eyes removed and placed in MSP buffer solution.

The buffer consists of a tablet of Sorenson's phosphate buffered saline solution ( $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ) at pH 7.2, dissolved in 150 ml of distilled water ( $\text{dH}_2\text{O}$ ). The addition of ten grams of sucrose in the 150 ml of buffer  $\text{dH}_2\text{O}$  brings the solution to a final volume of 200 ml. Ten grams of sucrose was added to the 150 ml of buffer and the final volume was adjusted to 200 ml with  $\text{dH}_2\text{O}$ . The purpose of adding sucrose is to increase the osmolarity of the buffer solution so it is slightly hyperosmotic relative to the photoreceptor cells. This causes the cells to shrink slightly, compressing the discs in the outer segments of the rod thereby decreasing the dead space and allowing more accurate absorbance measurements with the MSP. The buffer solution was mixed and stored at 4°C. Removing an eye from the buffer and placing it on a piece of filter paper, a #11 scalpel blade was inserted into the eye at the corneal-scleral boundary, making a hole.



Dissecting scissors inserted into this hole were then used to cut around the circumference, separating the eye into anterior and posterior halves. At this point the eye was placed back in the buffer. In the buffer, the lens was separated from the posterior half of the eye cup (if still present) and the retina gently separated from the rest of the eye cup. Once the retina was isolated and free from pigment epithelium, the retina was removed from the buffer solution to a glass slide.

The isolated retina was placed on a slide, and radial incisions were made in the retina using a scalpel so it would lie flat instead of in its natural, bowl shape. Then, two small square cover slips (18mm<sup>2</sup>) were laid on the slide, flanking the retina. Two drops of buffer were added to the prep and it was covered by a larger, rectangular cover slip (22mm x 30 mm) so that each end of the rectangular cover slip sat on top of a square cover slip.

### *Operational Procedures*

The measuring system consisted of a microscope, a video-capture box for image acquisition and storage, a background illuminator, a controllable strobe light, a camera, and a device that adjusts the gain and shutter speed of an image intensifier. The background illuminator was projected through a filter wheel containing five filters of the following wavelengths: 800 nm, 720 nm, 600 nm, 520 nm, and a blank allowing white light to pass through. The background illuminator was adjusted with an external power source, and was set between 1.0 amps (A) and 1.5 A. The voltage was adjusted to the lowest possible setting so the specimen could be viewed under the microscope, typically about 2.0 volts (V). The strobe light was used to flash a circular image onto the retina. This led to the formation of an optogram (Kuhne, 1878). The optogram is a latent image

visible on the retina due to the bleaching of visual pigment where the light hits, while the adjacent retina remains unbleached (Kuhne, 1878). The optogram I produced was a solid circle that occupied about a quarter of the microscope field. The strobe light was connected to a button, which when pressed, would flash the circular field a single time onto the retina. The strobe was used to test if contrast could be distinguished between the bleached and unbleached regions.

All setup procedures were conducted under 800 nm light, preventing any bleaching, yet allowing the retina to be seen on a video monitor attached to an intensified camera. After the baseline image was acquired under 800 nm light, the filter wheel was rotated to 720 nm. The retina was bleached by exposure to 720 nm light for about 1 minute before the next photograph was taken. This method was replicated for each filter.

#### L-THYROXINE TREATED RETINAE

##### *Preparation Techniques*

Under infrared illumination, retinæ were dissected free from the RPE and eye cup of enucleated eyes. Isolated retinæ were then placed in 50 ml vials with screw caps that were filled with media. The media consisted of Dulbecco's Modified Eagle's (3% Fetal Bovine Serum), Streptomycin and Penicillin (5,000 units/ml each) (Koyama, Horii, Miwa, Aizawa, 2003) and L-thyroxine at a concentration of  $1 \times 10^{-8} \text{M}$  (Wilt, 1959). Control media did not contain any L-thyroxine. All media were incubated at 28.5°C (Hightower, Renfro, 1988; Heller, personal communication), the optimal temperature for zebrafish. Sixteen vials were used and were divided into the following categories: Light-Control, Light-Thyroxine, Dark-Control, Dark-Thyroxine. The vials including specimens

to be kept in the dark were covered in aluminum foil to be completely light-tight. Each group thus contained four vials, one to be analyzed per day from each group.

### *MSP Retinal Preparation*

The retinae were dissected in the same manner as they were for 2D-imaging. Under infrared light, the retinal tissue was isolated from the RPE and eye cup and placed on a cover slip in a drop of MSP buffer solution. The retina was chopped into small pieces using #11 scalpel blades. This maceration increased the number of cells available for measurement by exposing edges of retina and liberating single photoreceptor cells. The sample was sealed on the cover slip with an overlying coverslip edged with silicone grease that sealed the preparation from evaporation and reduced movement of cells in the preparation. The sample was then placed on the stage of the MSP. A retinal edge is shown in Figure 2.1.

Figure 2.1. A retinal edge with rod photoreceptor outer segments exposed. This picture was taken using Nomarski differential interference contrast. See Gleyzes, Boccara, Saint-James (1991) for description.



### *The Microspectrophotometer*

The MSP is a single beam, computer controlled instrument that has been previously described by Loew and Wahl (1991) (Figure 2.2).

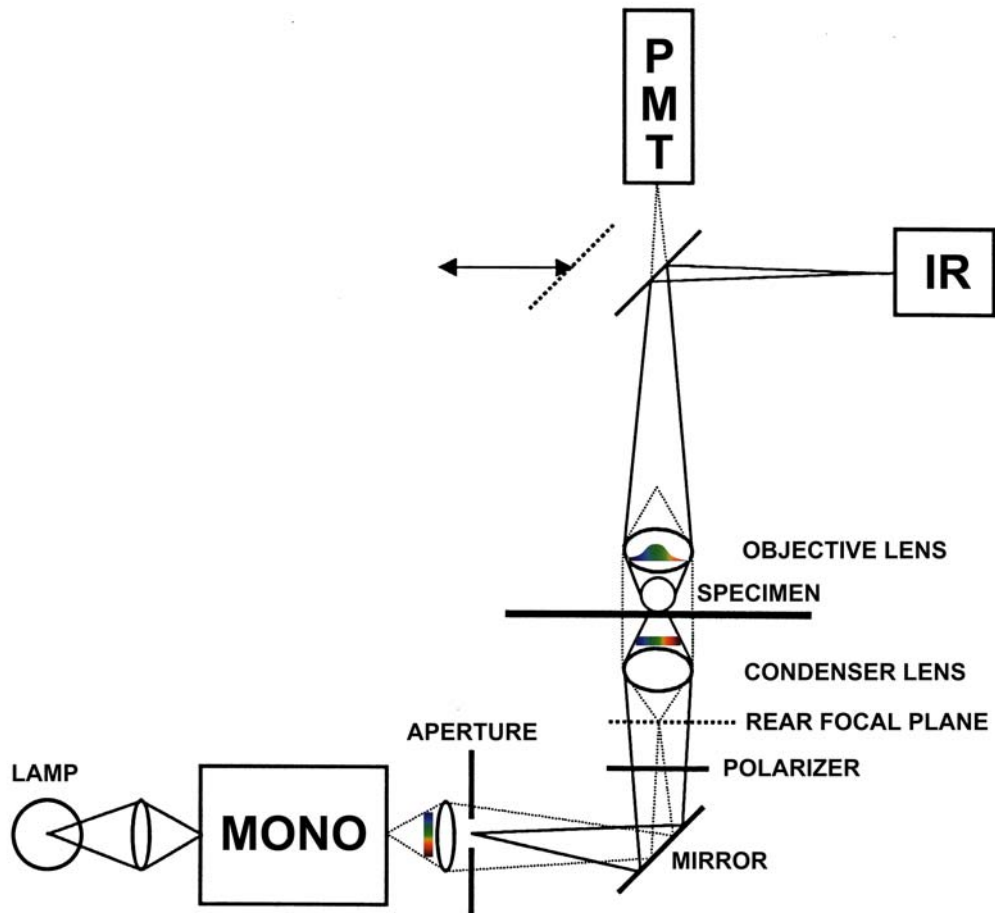


Figure 2.2. The Microspectrophotometer. PMT = photomultiplier tube. IR = Infrared-sensitive video camera. Mono = monochromator (Loew, personal communication).

Briefly, light from a stabilized quartz-halogen lamp is dispersed by a grating monochromator whose output wavelength can be selected by the computer program. This output is passed through a variable rectangular aperture forming a measurement beam, the image of which is brought to focus at the object plane of a microscope using a Zeiss Ultrafluar lens (40X, 0.45 NA). The object whose absorbance is to be measured is

moved over this beam by manipulation of the microscope stage position. The light from the beam is collected by a UV microscope objective (LOMO 100X, 0.90 NA) and directed either to an infrared-sensitive video camera, or a photomultiplier tube. An infrared background illuminator allows for the examination and orientation of the preparation.

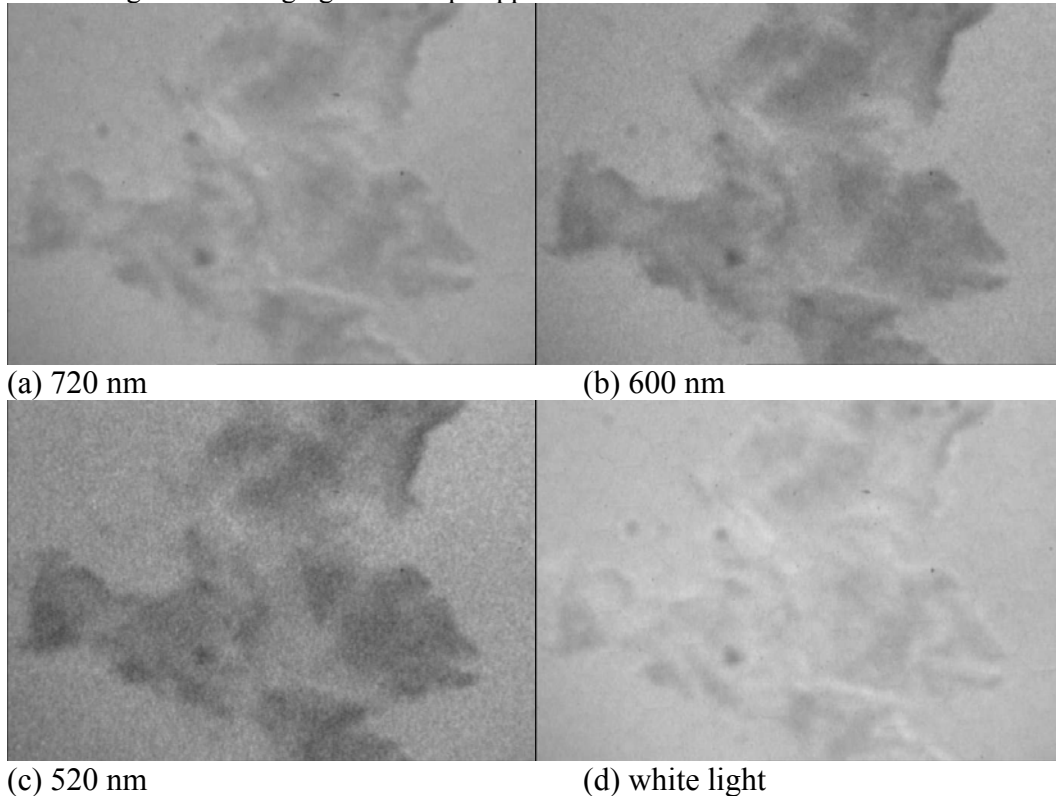
The MSP can be operated in two modes. The first mode (called continuous mode) scans the wavelength of the measuring beam from 750 nm to 350 nm and then back to 750nm at a rate of 100 nm/s. This allows for the plotting of an absorbance spectrum, a reflection of the difference between the baseline absorbance (set to zero by the program) and the absorbance of the cell at each wavelength. The second mode (single mode) sets the wavelength of the measuring beam and takes measurements at the selected wavelength at regular time intervals determined by the user. In both cases, measurements are first taken through a blank area of the preparation as baseline value. Measurements are then made of sample objects of interest. Absorbance is calculated as  $\log(\text{baseline}/\text{sample signals})$ .

## RESULTS

### 2D-IMAGING

The results from 2D-imaging did not reveal vitamin A<sub>2</sub> utilizing cells. Photos taken after each bleach did not show any difference in color, and thus bleached areas could not be determined from unbleached (Figure 3.1). In Figure 3.1, the same retina is viewed under light of the following wavelengths (in nm): 720, 600, 520, and white light. While the contrast among pictures is different, no differences could be seen in the retinae, which is necessary for determining the presence of any type of photoreceptor pattern in the retina. Since a vitamin A<sub>2</sub> utilizing cell will have a  $\lambda_{\text{max}}$  that is shifted towards the red (Bridges, 1972; Loew, 1995), the 720 and the 600 nm filters should show any cells that bleach at equal to or greater than these wavelengths. As previously stated, the LWS gene produces an opsin that when bound to vitamin A<sub>1</sub> is sensitive in the 490-570 nm region (Loew & Bowmaker, in press). If this opsin was bound to vitamin A<sub>2</sub>, it is expected to absorb in the 600 nm region. Thus, a difference would be seen between the 720 and 600 nm pictures. This was not the case.

Figure 3.1. Photographs of a zebrafish retina under four different light filters. These images were taken using the 2D-imaging microscope apparatus.

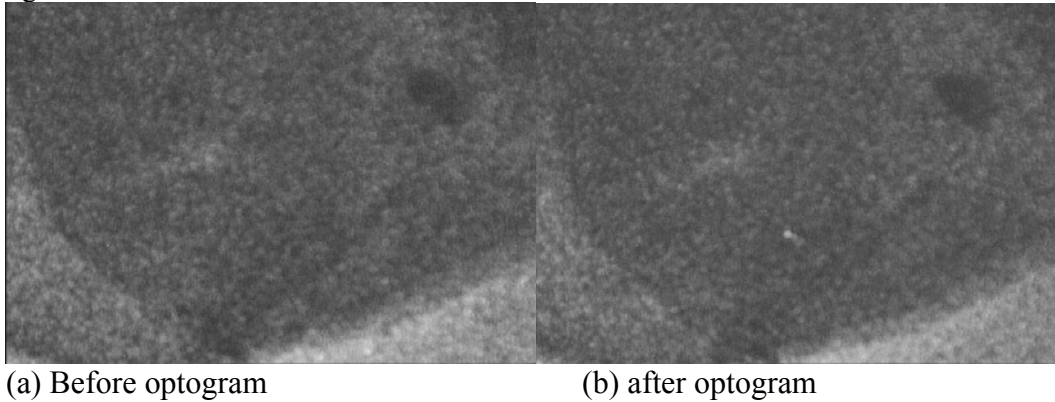


To test if the microscope apparatus was in fact bleaching, the strobe light was utilized for producing an optogram. A retina was placed on the stage in the same fashion, and one region of the retina was flashed with the strobe light about 10 times. The purpose of this was to be sure that the region in the field of the strobe light and no other parts of the retina would be completely bleached. After the strobing, the bleached and unbleached regions were still indistinguishable (Figure 3.2). This problem could have been due to a low optical density (Loew, personal communication). To determine if this was the problem, four retinas were stacked on top of each other, essentially multiplying the optical density of the single retina by four. The four retinae were bleached with the



strobe light in one region, a photograph was taken, yet there was still no visual difference. The integrity of the strobe light was then measured to see if it was, in fact, bleaching. A single retina was placed on the slide and bleached using the strobe light about 20 times. Then, the bleached region was dissected from the unbleached and MSP was conducted on each piece. The bleached portion showed mixed results. Some of the outer segments were bleached, but not all of them. The control showed a higher absorbance in the outer segments. Results from 2D-imaging were therefore inconclusive.

Figure 3.2. The optogram was projected onto the retina in the center of the field using a strobe light.



#### L-THYROXINE TREATED RETINAE

The thyroxine treated retinas showed no evidence of expressing vitamin A<sub>2</sub> in the photoreceptors. Rods provided the most reliable data because of their resiliency in the tissue culture over the course of the experiment. Two rounds of treatments were conducted on the retinae: the first was five days and the second, three days. The second culture was only three days because the quality of the retinas diminished so that few, if any, MSP measurements could be made. The results from the first treatment round are summarized in Table 1. The days in which no measurements could be made are noted on the table as “none”. The number of rods measured is noted in parenthesis after the  $\lambda_{\text{max}}$ .

Table 1. Average Rod  $\lambda_{\max}$  (nm) in Zebrafish (*Danio rerio*). Number of rods measured is shown in parentheses.

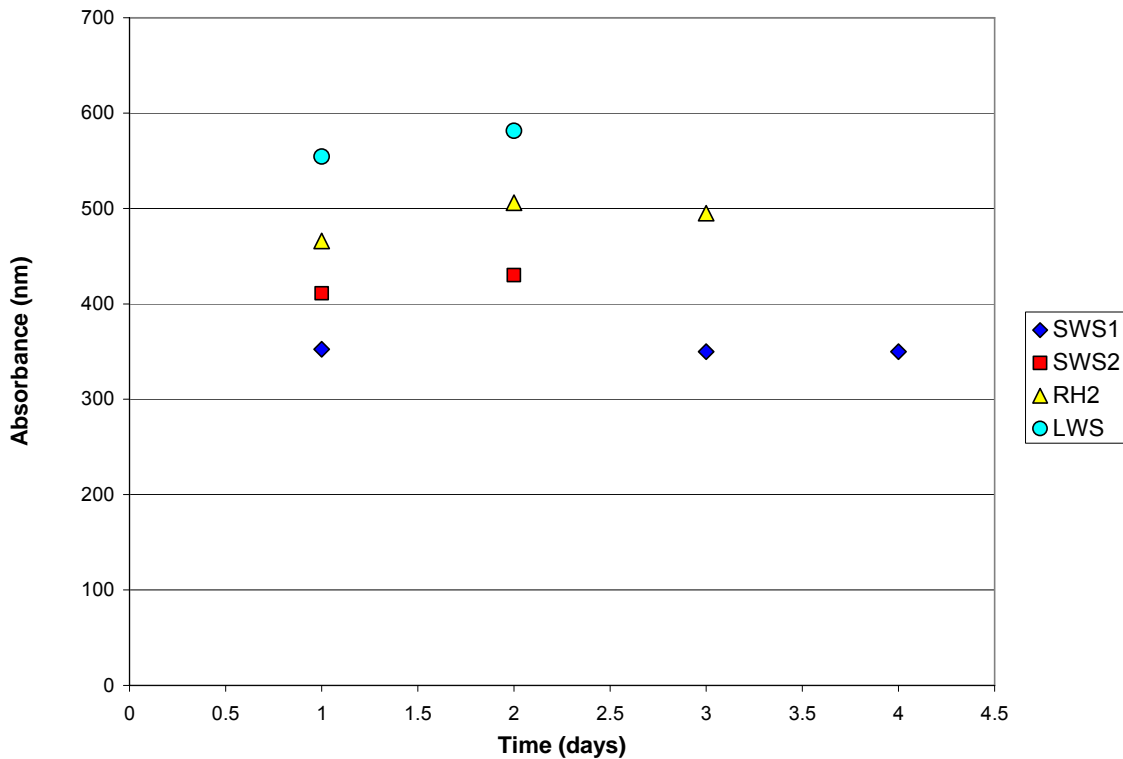
	Day 1	Day 2	Day 3	Day 4	Day 5
Light-Control	533.25 (2)	512.5 (2)	498.5 (2)	none	481 (1)
Dark-Control	489.42 (2)	none	502 (1)	505.67 (3)	none
Light-Thyroxine	502.38 (8)	500.45 (13)	504 (1)	507.5 (2)	none
Dark-Thyroxine	500 (7)	none	501.33 (9)	none	498.5 (4)

The light-control group showed an overall decrease in  $\lambda_{\max}$  over time, starting at 533.25 nm and finishing at 481 nm. The  $\lambda_{\max}$  of control rods is expected to remain the same over time, at about 505 nm. The dark-control retinae showed an increase in  $\lambda_{\max}$  from 489.42 nm to 505.67 nm, a 3.3% increase. The light-thyroxine group showed a slight increase in  $\lambda_{\max}$  over the course of the experiment, starting at 502.38 nm and after four days of thyroxine treatment the  $\lambda_{\max}$  was 507.5 nm, a 1.02% increase. The dark-thyroxine treated retinae showed an overall decrease in  $\lambda_{\max}$ . The initial average  $\lambda_{\max}$  was 500 nm and at the end of a five day treatment in L-thyroxine the retinae decreased to 0.3% to 498.5 nm.

Data obtained from cones in control media is summarized in Figure 3.3. Data from both light and dark control cones were combined in Figure 3.3. The  $\lambda_{\max}$  of the pigment produced by the SWS1 gene increased 0.71% from the first to the fourth day of MSP analysis. A larger  $\lambda_{\max}$  increase occurred in the SWS2 and RH2 pigments from the first to the fourth day: 7.06% and 6.22% respectively. The  $\lambda_{\max}$  of the LWS pigment increased from 554.425 nm to 581.5 nm from day 1 to day 2, an increase of 4.88%. Zebrafish have a  $\lambda_{\max}$  of 350 nm for SWS1 pigments while that of the SWS2 range between 407-416 nm (Loew & Bowmaker, in press). A  $\lambda_{\max}$  range of 467-505 nm is observed for RH2 pigment (Loew & Bowmaker, in press). There is some controversy

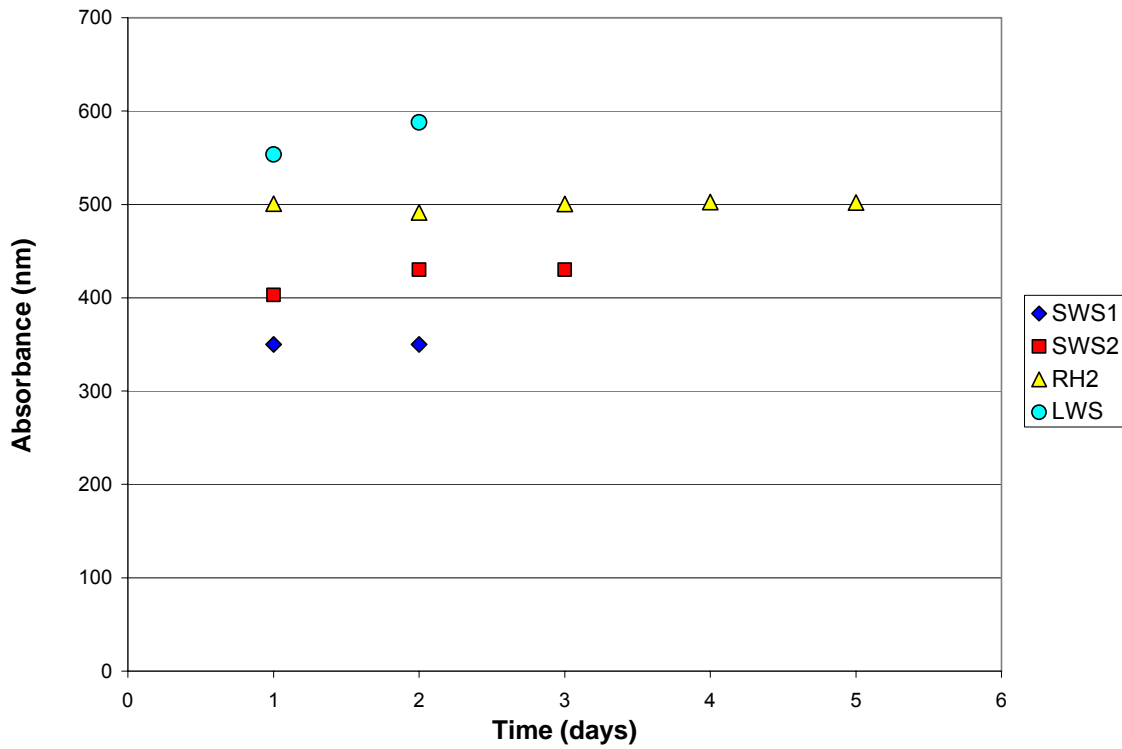
over the  $\lambda_{\max}$  of the LWS pigment: two genes have been found with  $\lambda_{\max}$  558 nm and 548 nm (Chinen *et al.* 2003), though there is no evidence of two distinct LWS cones (Loew & Bowmaker, in press). Some groups report the  $\lambda_{\max}$  of LWS cones to range between 556-570 nm (Cameron, 2002; Nawrocki, BreMiller, Streisinger & Kaplan, 1985; Robinson *et al.* 1993).

Figure 3.3.  $\lambda_{\max}$  of four classes of control zebrafish cones. The retinæ that these cones came from were incubated in control media. Each class of cone is designated by the legend and its  $\lambda_{\max}$  varies slightly over time. The absorbance of the cones was measured using MSP. The areas where points are missing are due to the fact that no usable data were obtained from the specimens on those days.



Data from both light and dark thyroxine treated retinæ are combined in Figure 3.4. The  $\lambda_{\max}$  of SWS1 cones remained constant from day 1 to day 2. Small increases were noted for the SWS2 and RH2 cones: 1.99% and 0.37% respectively. The LWS cones measured increased from 553.5 nm to 588 nm from day 1 to day 2, an increase of 6.23%.

Figure 3.4.  $\lambda_{\max}$  of four classes of L-thyroxine treated zebrafish cones. The retinae that these cones came from were incubated in media supplemented with L-thyroxine at a concentration of about  $1 \times 10^{-8}$  M. Each class of cone is designated by the legend and its  $\lambda_{\max}$  varies slightly over time. The absorbance of the cones was measured using MSP. The areas where points are missing are due to the fact that no usable data were obtained from the specimens on those days.



## DISCUSSION

### 2D-IMAGING

2D-imaging retains spatial information destroyed during tissue preparation for MSP.

Laying flat on a slide, the retina represents a 2-dimensional plane to work with. The purpose of this 2D-imaging paradigm is to selectively bleach the A<sub>2</sub>-containing photoreceptors while leaving unbleached A<sub>1</sub> photoreceptors, and to be able to detect the contrast from video images. For example, 500 nm light bleaches photoreceptors that have a  $\lambda_{\max}$  of 500 nm or greater, leaving photoreceptors with a  $\lambda_{\max}$  less than 500 nm unbleached. Another advantage of 2D-imaging over MSP is photoreceptors are not wasted because they are immeasurable. To measure the absorbance of a photoreceptor using MSP, the outer segment of the photoreceptor must be isolated or the tissue must be cut so that they are aligned on an edge (Figure 2.1). The beam of light can pass through isolated outer segment and the absorbance can be measured.

A disadvantage of 2D-imaging is that the optical density of the photopigments needs to be great enough to be able to visually distinguish bleached from unbleached tissue. Optical density is the absorbance of the photopigment. The photoreceptors must be positioned vertically, with their outer segments facing upwards, as oriented *in vivo*, in order to absorb light. If they are slanted, damaged, or if the outer segments were ripped off during retinal isolation, then the optical density will be too low to detect a contrast change after bleaching. An additional disadvantage is the resolution of the video image is not great; meaning large islands or regions of photoreceptors should be able to be identified, but cells that are in small islands or dispersed are not.

The difference between bleached and unbleached retinal tissue is apparent in some species with the naked eye. Kuhne (1878) describes the bleaching of the visual purple, or rhodopsin. The color of the tissue is originally a dull, pink-purple, but turns off-white when bleached. Kuhne (1878) observed this phenomenon in many different vertebrate retinæ. This contrast was not detected in the zebrafish retinæ after 2D-imaging was conducted (Figure 3.1). Visual analysis with the naked eye as well as video images failed to capture the color contrast.

In this experiment, video of the retina were taken before and after each bleach and analyzed using Adobe Photoshop. Stored video images were subtracted from each other, allowing for the detection of differences between two. Thus, subtracting an image of bleached retina at 500 nm from the completely unbleached baseline one (at 800 nm) theoretically would indicate the areas that were bleached by 500 nm light—the regions of the retina that are bleached by light equal to or greater than 500 nm would be visible using this type of analysis.

The filters that were used were: (in nm) 800, 720, 600, 520, and a blank. These filters were chosen based upon the hypothetical shifts in  $\lambda_{\max}$  that would occur when vitamin A<sub>2</sub> is utilized. The 800 nm and blank filters were needed as baseline and completely bleached comparisons. The 720 nm filter was chosen because of the belief that if a LWS cone was complexed with vitamin A<sub>2</sub>, the shift in  $\lambda_{\max}$  would fall between 720 nm and 800 nm. The 600 nm filter could bleach LWS cones if the  $\lambda_{\max}$  did not shift as greatly as predicted, or any long wavelength shifted RH2 cones. The 520 nm filter was used to detect any shifted rods. The filters would not be able to detect shifts in either

of the SWS gene opsins, regardless of the chromophore because of the minute shift that would occur.

The above analysis failed to demonstrate any  $A_2$  regions. After bleaching, there is no visual difference between the pre-bleached and post-bleached photos. Photo-subtraction fails to indicate any difference between the photos as well (Figure 3.1). These results support either of two probable conclusions: (1) the retina is not being bleached and/or (2) the optical density of the zebrafish retina is too small to detect bleached from unbleached zones. Optogram analysis paired with MSP of the retina indicates that the former conclusion is most likely not true. MSP of photoreceptors originating from the bleached portion of the retina (that which the optogram was projected onto) show equal or smaller absorbances than photoreceptors from the non-bleached portion, indicating partial bleaching had occurred.

Since contrast between bleached and unbleached regions of the retina could not be detected, four retinæ were stacked onto each other to multiply the optical density by four. The optogram was projected onto a portion of the specimen that was four layers thick, but still failed to produce noticeable bleaching with the naked eye. The problems with detecting contrast using 2D-imaging on zebrafish retinæ led to the failure of locating regions of vitamin  $A_2$  utilizing cells.

#### L-THYROXINE TREATED RETINAE

Since vitamins  $A_1$  and  $A_2$  are so similar in structure, an antibody does not currently exist that can distinguish between the two (Loew, personal communication). An extraction of all the metabolites of the retina would thus be inefficient in detecting vitamin  $A_2$ . Due to the similarities in structure, a different method must be performed to detect this specific

molecule. MSP exploits the differences between the  $\lambda_{\max}$ , providing a useful and efficient method of distinguishing between vitamins A<sub>1</sub> and A<sub>2</sub>.

In order for MSP to be effective, isolated photoreceptor outer segments with intact plasma membranes are necessary because they contain visual pigment and it is the absorbance of this pigment that is measured. After two to three days in the tissue culture, the cells did not show any absorbance because they lost most of their visual pigment. The short culture-life of zebrafish retinal tissue is unexpected because of the similarity to other fish tissue culture media that this experiment was modeled after (Koyama *et al.* 2003; Hightower & Renfro, 1988). According to Koyama *et al.* (2003), tissue culture flasks could be used for 10-20 days. Although the medium was not identical to that used by Koyama, I did not expect such different outcomes. The fact that control and L-thyroxine media both failed to preserve the quality of retinal tissue eliminates the possibility that L-thyroxine was harmful to the tissue. Past experiments by Wilt (1959) and Beatty (1968) with vertebrate eyes and L-thyroxine avoided the problem of a suitable medium because they used thyroxine-cholesterol pellets implanted in the eye and intraperitoneal thyroxine injections, respectively. These procedures do not result in animal death, eliminating the need for a culture.

A protocol was not able to be obtained allowing for the performance of surgical acts on live zebrafish and the ability to store them in the lab for recovery and analysis. As a result, the fish used in this experiment had to be euthanized within 8 hours of retrieval. A tissue culture of the retina proved to be the only way to treat retinae for a duration long enough for vitamin A<sub>2</sub> to be synthesized. The major problem encountered in this experiment was the degeneration of retinal tissue in the media over time. The



media could have been infected with bacteria immune to penicillin or streptomycin (the two antibiotics used in the medium), or infected with a non-bacterial agent. Thus, sterilized media and instruments could allow for the increased lifetime of the retinal tissue culture. Oxygenation of the media could have been another source for degeneration. After two or three days, the oxygen levels could have been too low for tissue metabolism. An observation that was made in three or four day old tissue was the swelling of photoreceptors. The osmolarity of the control and L-thyroxine stock media was 300 mOsm, which is suggested for tissue culture (Loew, personal communication; Beyenbach, personal communication). The pH of the stock media was 7.43. Since the cells degenerated over time, it is possible that after two or three days the osmolarity or pH changed due to some unknown factor. The pH and osmolarity of the media containing degenerated tissue were not measured in this experiment, but is recommended in the future.

The reason no vitamin A<sub>2</sub> containing cells were found was that not enough cells were measured, because they were too deteriorated. When a method that sustains zebrafish retinal tissue in a culture for five days is developed, then the methods performed in this experiment should be sufficient to detect cells that use vitamin A<sub>2</sub> in their rods and cones. This time period should allow large (and therefore detectable) numbers of photoreceptors to utilize vitamin A<sub>2</sub>. Ultimately, when the presence of vitamin A<sub>2</sub> is confirmed in the retinae of zebrafish, it will add momentum to the search for the hypothesized dehydrogenase that converts vitamin A<sub>1</sub> to A<sub>2</sub>.

The results do not lead to any conclusion about the presence of vitamin A<sub>2</sub> in the retinae of zebrafish, because of the inability to culture retinas for a duration long enough

to obtain large numbers of vitamin A<sub>2</sub> utilizing photoreceptors. According to Loew (personal communication) it can take anywhere from two to five days for vitamin A<sub>2</sub> expression to occur. The media used in this experiment is modified from other fish cell cultures. Retinal tissue was preserved in a quality sufficient for MSP analysis only for about two to three days. Although this duration should allow for the detection of the vitamin A<sub>2</sub> chromophore in zebrafish retinae, these specific cells were not found. MSP analysis measures the absorbance of photoreceptors. The most dramatic shift in  $\lambda_{\max}$  that occurs when the vitamin A<sub>1</sub> chromophore is replaced with vitamin A<sub>2</sub> is found in long wavelength sensitive pigments (Loew, 1995), i.e. the LWS cones of zebrafish. A larger shift change is more noticeable in the LWS cones than either of the SWS or the MWS, although a shift would still occur. Analyzing the LWS cones with MSP allows for the distinction to be made between cones utilizing vitamin A<sub>2</sub> and cones that have varying maximum absorbances. Thus LWS cones were the major target for MSP analysis in this experiment. Unfortunately, these cones proved to be highly elusive in retinae that were several days into L-thyroxine treatment. Cones in general did not survive as long in the medium when compared to rods. There were only two LWS cones found in retinae that were treated in L-thyroxine for two or more days. LWS cones were found in large numbers in control retinae that were analyzed on the same day as euthanasia. Seven MWS cones were recorded after at least two days in L-thyroxine. Rods were abundant throughout the course of the experiment, yet showed no evidence of a shift in  $\lambda_{\max}$ .

According to Table 1, no increase in  $\lambda_{\max}$  was noted for zebrafish rods. The number of rods measured on days four and five are not great enough for reliable and consistent data to be obtained from them. Zebrafish rods have a  $\lambda_{\max}$  of about 507 nm

because this is maximum absorbance of rhodopsin with the vitamin A<sub>1</sub> chromophore (Reeves, 2004; Bridges, 1973). With a vitamin A<sub>2</sub> chromophore, the  $\lambda_{\text{max}}$  shifts to about 523 nm (Bridges, 1973). Results from L-thyroxine treated retinae are consistently averaged closer to the  $\lambda_{\text{max}}$  of vitamin A<sub>1</sub>. There is some variance among the retinae in Table 1, which could be due to idiosyncratic differences among rods or inaccuracy/inconsistency with the baseline.

Figure 2.1 shows the change (or lack there of) in the four cone classes of zebrafish. The results show the  $\lambda_{\text{max}}$  remains relatively constant over the control and L-thyroxine treatments. The control is expected to remain constant, while the L-thyroxine treated tissue is expected to shift to a longer  $\lambda_{\text{max}}$ . The shift would be most dramatic in LWS cones (Loew, 1995), but too few were measured with MSP in this experiment to support this conclusion. While there is a shift towards a longer wavelength in the LWS cones in Figure 2.2, the same shift is reflected in the control data shown in Figure 2.1, suggesting that the shift is due to variance within the zebrafish population that was analyzed. RH2 cones showed a similar consistency in its  $\lambda_{\text{max}}$  between the control and L-thyroxine tissue, as did the SWS2 and SWS1 cones.

It is obvious that more work must be done with zebrafish photoreceptors to prove the existence and location of vitamin A<sub>2</sub>. In these experiments novel techniques were used to try to accomplish these objectives. 2D-imaging is a useful and insightful technique for locating specific regions of the retina based on absorbance. MSP has been used for many years and will continue to be used because of its proven success in measuring absorbance spectra of photoreceptors. Using the results from this experiment, an adapted medium for tissue culture could be used to preserve thyroxine treated retinae,

giving us information that zebrafish contain an enzyme that can convert vitamin A<sub>1</sub> to A<sub>2</sub>.

This can be proved with MSP, and when 2D-imaging is applied these regions can be

localized and then targeted for further MSP analysis.

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