Colors Underwater: A Behavioral and Neural Systems Study on Color Vision in the Larval Zebrafish

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Colors Underwater: 
a behavioral and neural systems study on color vision in the larval zebrafish

A dissertation presented
by
Drago Andres Guggiana-Nilo

to
The Committee on Higher Degrees in Biophysics

in partial fulfillment of the requirements
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in the subject of
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Being able to see color is such a natural and intrinsic component of our vision that we rarely pay it mind. In fact, color vision is a very widespread capability of visual systems across species, but despite its intuitive nature for humans, its neural implementation is not well understood in any organism. The zebrafish, *Danio rerio*, has potential as a color vision model organism, given its optical accessibility, established genetic toolset and four types of cone photoreceptors, all in a vertebrate system. Despite these advantages, very little is known about the color-related computations taking place in the zebrafish brain. Hence this work aims to shed light on these processes, both from a behavioral and neural perspectives. In the first study, a phototaxis assay was used to assess the wavelength preferences of the zebrafish larva. Stimuli were projected from below following the animal in a closed loop, and forcing it to make a choice at every turn between the stimulus on the left or the right. This study showed that zebrafish larva avoid UV light, especially if combined with visible light. This UV avoidance behavior is dependent on the eyes, and in particular on the presence of the UV cone in the retina. The true evolutionary drive for such a behavior is unknown, but it is suggested that its role is to balance sun exposure with enough light to find food and shelter. The second study delves into the brain of the larva, and asks whether there is any indication of color computations taking place there. In particular, the study focuses on the interaction between the output of the eye, the retinal ganglion cells (RGCs) and...
their main target brain region, the optic tectum (OT). The study found that the responses at both regions seem to be UV dominated, as expected from previous reports, and that some of the cone types are correlated in generating RGC or Tectal responses. This is an indication of RGC color types, a staple of the known color vision systems. Additionally, there are clear signs of chromatic information processing, as there is input decorrelation between the two cell populations. This is observed more markedly in terms of chromaticity of the stimulus than in terms of its spatiotemporal pattern. In sum, it appears the zebrafish has the potential to become a color circuitry model organism, given it performs wavelength specific behaviors and it shows indicators of color information processing, even in the absence of learned behaviors. This study sets the first steps in the establishment of this model, and paves the way for the next stages in fully elucidating the color vision system of a vertebrate.
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Le dedico este trabajo a mi familia, y en particular a mis padres, Karin y Drago, porque desde mi primer día en este mundo me lo han dado todo sin dudar un segundo.
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The work in this thesis stands on the shoulders of a large body of vision and zebrafish research. Hence, this section will put the ensuing chapters within the larger framework of these fields. In particular, the overall structure of the visual system will be briefly introduced. This will be followed by luminance, motion and color vision, general subfields of vision research. The next section will deal with the use of zebrafish as a model organism for neuroscience, and the properties that make it ideal for this role. Finally, the last two sections will cover the main methodologies utilized in this research, namely the combination of 2 photon microscopy and functional calcium imaging, and the analysis of neural population data.
1.1 General organization of the visual system

Design features of sensory systems

There are several aspects of the visual system that are shared between most sensory modalities, and it is helpful to discuss these in a more general context so that the specifics of the visual system do not overshadow its purpose as a sensory input. Three of those aspects will be covered here: physical signal transduction, receptor location and tuning properties.

All sensory systems have the particularity that, despite being embedded in a biological entity, they have to interface with the physical world, extract information from it and convert it into a format that can be utilized by the rest of the organism \[1\]. This role is taken up by the sensory receptor cells for each sensory system, including the olfactory receptors for olfaction, mechanoreceptors for touch and the photoreceptors in the eye \[1\]. All of these cells express proteins that are sensitive to their respective stimuli, but manage to capture some of the information contained in these stimuli and they translate it into biochemical signals via transduction cascades \[1\]. The visual cascade will be discussed in more detail below, but there are several parallels between it and the other modalities.

Another highly conserved feature of sensory systems is the specialization of the organs containing the receptors to optimize the capture of the stimuli \[2\]. In the case of the visual system this role is fulfilled by the eye and its light collection optics, in olfaction by the nose and olfactory epithelium and in taste by the tongue \[1\]. All of these organs are able to physically optimize the concentration of stimuli around the receptors and hence enhance the extraction of information \[2\].

Finally, all sensory systems work by using receptors tuned to different ranges of the stimulus space \[3\]. These ranges, termed receptive fields, allow sensory systems to extract features from stimuli differentially, and also then encode more complex features of the stimulus even when they are highly compressing the information into biochemical signals \[3\]. In vision, receptive fields have both a temporal and a spatial component, since different cells will have access to different parts of the visual space \[3, 4\]. On top of this, most cells will be tuned to different aspects of the stimulus
such as motion direction, spatial frequency or color [5]. All of these will be discussed in more detail below.

**Light collection by the eye**

As mentioned above, a common feature within sensory systems is their reliance on specialization of the organs that house them [1]. Although across the animal kingdom eyes have several designs optimized for different functions *ad hoc* to the ethology of the animal, they are all optimized for the collection of light [5, 6]. There are two main types of eyes: composite and non-composite.

The former are seen in invertebrates, and are formed by many individual eyes that cover a different fraction of the visual field [7]. The latter, like the human eye, have a single eye structure that collects light from the environment via a lens and focus it into a highly specialized light sensitive tissue called retina [6]. The following section will focus on the vertebrate retina and its general principles. The invertebrate structures will be briefly covered in later sections.

**Retinal organization**

In vertebrates, the main visual sensory tissue is the retina (see Figure 1.1.1 for an overall schematic). This layered structure has a very stereotypical organization, with a photoreceptor layer that converts the incoming photons of light into neural potentials [5, 8]. These graded signals are then further processed by the bipolar and ganglion cell layers and are finally relayed to the rest of the brain for subsequent stages of processing [8]. The incoming sections will deal with each one of these stages in more detail.

Photoreceptors are the core of visual systems. These cells are able to convert light into chemical energy by expressing specialized proteins called opsins [8]. These proteins (rhodopsin in particular) were first identified and characterized by Franz Boll and Wilhelm Kuehne in 1876 and 1878 respectively [9], and are transmembrane proteins with an internal binding site for the chemical retinal. This binding site has a high affinity for the *cis* form of the compound, which transitions to its *trans* form upon receiving energy from photons of a particular wavelength range [5]. The
affinity for the trans form is lower and hence it is released from the molecule [1]. In the dark, photoreceptors are constantly depolarized and releasing glutamate, but upon triggering of the opsin photo-sensing reaction, G-proteins trigger a biochemical cascade that ends in hyperpolarization of the membrane and suppression of glutamate release [8].

Sensing of photons by the opsin protein is dependent on the absorption spectrum of the protein-retinal complex [8]. Depending on the biochemical environment around retinal, the absorption spectrum of the complex will vary across a wide range of wavelengths, both within and outside the visual range of humans [5]. In particular, Nathans and Hogness [10] were able to clone the genes encoding for the human opsins, and identified the sequence differences that give each protein its own absorption spectrum. Since then several opsins have been isolated from different species, yielding more insight into the connection between the absorption spectrum of the protein and the sequence changes required to yield that spectrum (see Suga et al. [11], Davies et al. [12], Kitamoto et al. [13] for examples, and Shichida and Matsuyama [14] for a review). Later sections will cover the way these different types of opsins are used as the basis of color vision.

Anatomically, photoreceptors are also highly specialized since they concentrate expression of the opsin protein in an elongated portion of the cell termed the outer segment [5, 8]. Then the biochemical cascade transmits the signal to the inner segment and cell body of the cell, locus of the nucleus and the connections of the photoreceptor to other cells [8].

Signals are relayed from the photoreceptor into two general types of cells: horizontal cells and bipolar cells [1]. As their name suggests, horizontal cells are not connected in a top down organization, but instead connect different cones together laterally, both in a color dependent and color independent manner [8]. These cells are thought to participate in the generation of the surround in receptive fields and also in some color phenomena (such as color constancy) that will be explained below [1, 15]. Bipolar cells on the other hand do have a top down organization: They propagate signals from the photoreceptor layer to the ganglion cell layer [8]. This signal propagation also involves a degree of processing, since bipolar cells pool inputs from a number of photoreceptor cells, the nature of which depends on the organism, location on the retina and other factors still under investigation [5]. Additionally, bipolar cells cluster into two distinct types based on the kind of synapse they have with photoreceptors. OFF bipolar cells become active in the absence of light,
Figure 1.1.1: General structure of the retina After light hits the eye, it is focused on the retina by the lens. Light goes through all the layers of the retina until it is absorbed at the outer segments of the photoreceptors. These cells then relay the signal to bipolar cells, while being under feedback regulation from the horizontal cells. Bipolar cells then further relay the information pooled from several photoreceptors into the retinal ganglion cells. Amacrine cells collaborate in the computation by providing inhibitory and excitatory inputs to bipolar and ganglion cells. Processed visual signals then leave the retina via the retinal ganglion cell axons, forming the optic nerve. Adapted with permission from EA Kimbrel and R Lanza. Current status of pluripotent stem cells: moving the first therapies to the clinic. Nature Reviews Drug Discovery, 2015. doi: 10.1038/nrd4738

and they do so by making ribbon type synapses with the photoreceptors [5, 8]. These synapses follow the glutamate release of the photoreceptors and hence become inactive in the presence of light and active in its absence. On the other hand, ON bipolar cells make sign inverting synapses with photoreceptors, so that hyperpolarization of the photoreceptor generates excitation of the bipolar cell via metabotropic mechanisms [1, 8]. Lastly, interacting with bipolar cells and retinal ganglion cells (RGCs) are amacrine cells. These cells’ roles are not fully clear, but they have been involved in the generation of direction selectivity [16]. They can regulate the output of RGCs via inhibitory and excitatory synapses to cell bodies, and also into shunt synapses at the bipolar-RGC synapse [8].

The last layer of the retina, the ganglion cell layer, adds an extra stage of processing and input
collection before relaying signals to the rest of the visual areas in the brain [2]. Some of the more complex properties of visual systems can be found already at this layer, such as direction selectivity and color opponency [15]. This layer has been the subject of extensive research since it is the output layer of the eye, and the axons of the retinal ganglion cells form the optic nerve [18]. These cells have been classified in several groups based both on their morphology and also on their functional properties [18, 19]. Among these are tuning for orientation and direction of motion, spatial frequency, edges, colors and other properties of visual scenes [20–22].

The signals that leave the retina reach conserved regions in vertebrates [23–25], although there are several variations depending on the species, some of which will be described below.

**Intermediate visual areas**

Once signals leave the retina, they are relayed to a structure termed Superior Colliculus (SC) in mammals and Optic Tectum (OT) in other species (for reviews see Gandhi and Katnani [26], Knudsen [27], Butler and Hodos [28]). In mammals they are also relayed to a structure called the Lateral Geniculate Nucleus (LGN) in the thalamus. The latter structure, once thought to only be a relay station on the way of signals to the tectum, has been shown to accomplish more complex functions, especially given the large amount of feedback projections it receives from the visual cortex [1]. See Figure 1.1.1 for a schematic of the pathway.

The OT is a highly conserved structure, found all the way from fish to reptiles and up to primates and humans [1]. This structure contains a very detailed retinotopic map of the visual field in its superficial layers, namely, areas sensitive to adjacent points in the visual field are also located adjacent to each other in the brain [29]. Underneath the visual layers, the OT has mixed modality layers that receive input from other senses and also have motor-related outputs. The rationale behind this is the role of the OT in orienting behaviors and attention [27]. Functionally, when a stimulus calls the attention of the animal, the overlap of the dorsal retinotopic map and the ventral motor map helps in generating saccades and orienting movements towards this stimulus [27]. Extensive research in primates has show that this region is also involved in attention itself, since via electrical microstimulation one can modulate the fixation on a trained target when other confounds are present, but
Figure 1.1.2: Visual pathway in primates  Simplified diagram of the primate canonical visual pathway. After visual signals leave the eye through RGC axons, they synapse either at the LGN or the SC. Targets at the LGN then synapse into V1, which then projects to higher visual areas. SC in turn projects to the PU, which then projects to the dorsal pathway. LGN is lateral geniculate nucleus, PU is pulvinar, SC is superior colliculus, V1 is primary visual cortex, hMT is medial temporal area. Adapted with permission with permission from Strand-Brodd, K, U Ewald, and H Grönqvist. Development of smooth pursuit eye movements in very preterm infants: 1. general aspects. Acta Paediatrica, 2011. doi: 10.1111/j.1651-2227.2011.02218.x

not in their absence, i.e. when attention is not required [27, 29]. Its function in other vertebrates, like fish, will be discussed in more detail below, but in brief, it is less well defined, although the orienting and multi-modal character of it remains [30].

Higher visual areas

Most of the complex functions of the visual system in mammals are accomplished in different areas of the visual cortex. Signals are received from the LGN into area V1 and then further processed in subsequent stages [2]. Understanding of the specific function of these areas is still in progress, but here one can find cells sensitive to faces in general, to specific faces or elements, to the same element in several different orientations and many other examples of highly complex operations [5].
In particular, information seems to split in a dorsal pathway, involved in motion and location of objects, and a ventral pathway, involved in object and face recognition [1]. Accordingly, receptive fields get increasingly more complex as visual information moves through these areas and eventually engulf most of the visual field [5]. The current view is also complicated by the large amount of feedback projections in all of these areas, so that the system cannot be analyzed as a simple feedforward network in series. Further discussion of these areas is beyond the scope of this work, but the interested reader is invited to follow the references listed [5, 32–34].

1.2 Luminance and motion vision

The preceding sections cover the overall anatomical and physiological features of the visual system. The following sections will instead treat its functional aspects. In particular, this section will deal with how the visual system detects and signals the presence of light, and how these signals are used for the detection of motion and its directionality.

ON/OFF pathways

As mentioned above, the signals from photoreceptors measure the amount of light in the form of different degrees of hyperpolarization [8]. This represents a conundrum, since the absence of light has a direct signal (constant release of glutamate from the photoreceptor, dark current), but the presence of light is represented by an absence of signal [5]. Hence the visual system across many species performs an essential operation in the early stages of vision: the generation of an ON and an OFF streams of signals. As hinted above, the OFF signal comes from the ionotropic glutamate receptors in OFF bipolars that generate depolarization during darkness. The ON signal instead comes from metabotropic glutamate receptors in ON bipolars that trigger a sign inversion of the signal, so that there is depolarization of the bipolar cell membrane when the glutamate stream from the photoreceptors stop [8]. These two streams are then combined in different manners in the ganglion cell layer with the help of amacrine cells, generating ON, OFF and ON-OFF responses in RGCs. This allows the organism to extract light increments, decrements, and also spot size from
the visual field already at the retinal level \([5, 8, 16]\).

The other relevant component of these parallel streams is that, through their combination, spatial receptive fields can be generated. Since ganglion cells can pool their inputs from a mixture of ON and OFF bipolars, including lateral inhibitory inputs via amacrine cells, they are the first stage of vision that can construct spatially complex receptive fields with center-surround structure \([5]\). This refers to the excitation of the cell by stimulating the center of the receptive field with one signal (i.e. the ON signal), and the inhibition of the cell by stimulating the surround with the opposite signal (OFF in this example). These cells have been proposed to be the building blocks for edge detectors in the visual pathway, and can be found in all vertebrates surveyed to date \([1, 3, 16]\).

**Motion vision and direction selectivity**

Detection of luminance alone allows for parsing static images, and further processing allows for extraction of several complex features from them. The missing element is the time dynamics of real scenes, especially with respect to the detection of motion, an essential component of visual systems due to its evolutionary relevance.

The most important discovery regarding the perception of vision was the finding of direction selective cells in the retina. These cells have a receptive field like other ganglion cells, but their responses are triggered by motion within this receptive field and in a particular direction. They were discovered by Hubel and Wiesel \([4]\) in the cat and then found in several other organisms, including rabbits, fish and even flies \([16]\). The majority of these cells seem to be of ON-OFF nature, but there are also ON and OFF direction selective cells, depending on the organism. The output from these cells is combined in a non-linear fashion at the visual cortex, so that cells with large, elongated receptive fields can be found there \([35]\). These cells offer the machinery to both, compute motion of objects in a scene, but also compute self motion in the environment when combined with motor reaference and other sensory cues.
1.3 Color vision

Like motion, color is an extra layer of information that can be extracted from visual scenes. In this case, instead of correlating images in time, color relies on a different percept generated as a function of the wavelength of light sensed, as opposed to only the intensity of it.

Properties of color vision

Before delving into the mechanisms leading to color vision, it is important to be aware of the properties human color vision has, since these will lead to testable hypotheses for the color vision of other organisms.

The first feature to mention is the principle of univariance, which is more of a physical principle than a property. This states that each individual photoreceptor is actually color blind, and the extraction of information from a visual spectrum comes only from the signal comparison between photoreceptors with different wavelength sensitivities [36]. This is because the output of a single photoreceptor is just a photocurrent, which is sensitive to both the wavelength and the amount of light, and hence neither of them can be discerned from the other unless a comparison is made with a photoreceptor receiving the same amount of light but sensitive to different parts of the spectrum. The end result of this principle is that the perception of a particular color can be replicated via many combinations of wavelengths, as long as those combinations all excite the different types of cones in the same pattern [37] (Figure 1.3.1).

Second is the idea of color contrast. This refers to the enhancement in the contrast of an image based on the wavelength of the light illuminating it and the wavelength of the light it absorbs. In particular, this contrast mainly depends on how the combination of wavelengths of adjacent parts of the image interact with the cones in the eye. If they excite cones differentially, then they will appear with very high contrast, even if they are perfectly matched in terms of luminance [39, 40].

Third is the complement of color contrast, the phenomenon of color constancy. This consists
Figure 1.3.1: Principle of univariance. Diagram depicting the instantiation of the principle of univariance. Given the cone absorption spectrum shown (absorption in the y axis and wavelength on the x axis), if excitation is at $\sim 450\text{nm}$ or $\sim 630\text{nm}$ the output of the cell will be identical, hence making the cone color blind. Adapted with permission from Jeremy M Wolfe, Keith R Kluender, Dennis M Levi, Linda M Bartoshuk, Rachel S Herz, Roberta L Klatzky, Susan J Lederman, and Daniel M Merfeld. *Sensation & perception*. Sinauer Sunderland, MA, 2006
on the relative independence of the wavelength of illumination and the color of an object. In general, the visual system is extremely robust when generating the perception of color of an object, regardless of whether it is viewed in very white or very orange light, as would be the case at noon versus at dawn or dusk in a sunny day. This has clear evolutionary advantages, since otherwise our perception of the color of any object would be extremely dynamic based on the lighting conditions [41]. This is also obvious when looking at the large amount of color adjustments digital cameras have to make, since CCD chips do not have color constancy natively and hence will report colors differentially depending on the light source [42].

**Cellular basis of color sensing**

There are several systems at play in the generation of the properties outlined above. As explained, the principle of univariance is a physical consequence of the process of phototransduction. Hence, color vision requires intrinsically the mixture of color signals from different cones to be able to calculate differences in wavelength within a particular scene. In humans and primates this mixture is done at the level of the bipolar and ganglion cells, where different bipolar cells will feed more or less dedicated channels to ganglion cells, which effectively take the difference between the red-green and blue-yellow channels [35, 43]. These fundamental channels are propagated to the higher areas like the LGN and visual cortex, where further computations are thought to take place and generate the hues we are able to perceive [44].

There is a subset of RGCs that show color opponent receptive fields, namely they are excited by light from one wavelength range and inhibited by light from a different wavelength range (either in a center only or center-surround organization, 1.3.2 (Left)). These cells would allow for the discrimination of colors, but to be able to calculate color contrast or constancy, they are missing inhibition of the response caused by the non-excitatory color (i.e. they would need areas with concomitant +red and -green, as opposed to just +red) [45]. This is precisely the structure of the receptive fields of double opponent cells in V1. These cells have a center that is excited by one color range and inhibited by the rest of the colors, and a surround that is inhibited in the same fashion (Figure 1.3.2 (Right)). Hence they are intrinsically able to generate color contrast if the bound-
Figure 1.3.2: Single and double opponent receptive fields (Left) Schematic of the inhibition and excitation profiles for a single color opponent cell in one dimension. +L represents red cone-mediated excitation, and -M represents green cone-mediated inhibition. This gives rise to a cell that is sensitive to red light in particular, but cannot generate color contrast on its own. (Right) Excitation and inhibition one dimensional profile for a double opponent cell. L represents contributions from the red cone and M contributions from the green cone, + is excitation and - is inhibition. With a receptive field like this, a cell can calculate color contrast if a red/green edge aligns with the receptive field. Adapted with permission from SG Solomon and P Lennie. The machinery of colour vision. *Nature Reviews Neuroscience*, 2007

Evolution of color vision and pigments

The evolution of color vision is a complex question given the many components of color vision systems. The start of the discussion has to commence with the evolution of photopigments, since they are the input layer to any color vision system.
There are currently five large families of visual opsins in vertebrates (SWS1, SWS2, RH2, LWS and RH1) and some additional ones in invertebrates. The origin of the first photopigments is unclear, but it seems to be at the level of green algae several millions of years ago [51]. After this first photopigment, a gene duplication must have allowed the copy to start accumulating sequence changes to that a mid/long wavelength sensitive and low wavelength pair could coexist in the same organism [52]. In vertebrates the pigments kept diverging so that eventually the five families of pigments appeared, with the rod opsins (RH1, for rhodopsin-like 1) being the last addition to the set [53]. In particular, rod opsins appeared at some point after the appearance of the jawed fish, although the other four families were present already at the level of jawless fish. This also establishes that ancestral fish had a large array of cone opsins, and hence some must have been lost in the transition to terrestrial organisms [54, 55]. Even within fish there is a tremendous variation in the number and identity of the opsins being expressed. Deep sea fish lack most cone opsins [56], but fish like the african cychlid have several opsin genes that they can express selectively depending on their environment [57](Figure 1.3.3).

In terrestrial vertebrates there is also a lot of variation in terms of opsin genes. Amphibians and reptiles kept different subsets of the ancestral genes, but they are normally tri- and tetrachromatic, respectively [7, 59]. The SWS1 (for short wavelength sensitive 1) gene was actually lost in birds and then regained later via convergent evolution, either centered on the violet or UV (ultraviolet) ranges [53]. Mammals, due to their early nocturnal nature, lost most of the opsin genes, so that with the exception of humans and Old World monkeys, most mammals are dichromats with just an LWS (for long wavelength sensitive) and SWS1 cones. The SWS1 cone mutated to a blue sensor in some species, such as humans, and the LWS gene must have undergone a duplication to generate the MWS (for medium wavelength sensitive, part of the LWS family) gene after several rounds of evolution [58].

Other relevant components of color vision are the different cell types (and underlying circuitry) that participate in the generation of color sensation. Evolutionary history at this level is a much harder question, since neither of the components has a uniquely defined sequence that can be compared, but instead there are several possible molecular signatures for each. Current evidence proposes that one step in this evolution are double cones. They are thought to be ancestral forms of
Figure 1.3.3: Phylogenetic distances between opsin families  Tree with the phylogenetic distances (defined as aminoacid substitution rates) between the five opsin families in vertebrates, LWS, SWS1, SWS2, RH1 and RH2. Within each clade there are representative vertebrate species indicated. The L, M, M1, S, S1, S2 and UV letters indicate different center absorption wavelengths of the respective pigments. Adapted with permission from Gerald Jacobs and Mickey Rowe. Evolution of vertebrate colour vision. Clin Exp Optom, 87(4-5):206–216, 2004. ISSN 1444-0938. doi: 10.1111/j.1444-0938.2004.tb05050.x
single cones in the middle of differentiation. Additionally, horizontal cells seem to be conserved in terms of function and connectivity in terrestrial vertebrates and hence would have originated in their fish ancestors [58].

As mentioned above, invertebrates took a highly parallel road in their development of a visual system, so that most of the cellular structures are very different to the vertebrate ones, and the opsin proteins bear less similarity [7]. Regardless, there are several invertebrates with very rich sets of opsins and functional color vision systems, meaning the evolutionary drive for color vision was strong enough to drive such high convergence [7].

**Comparative color vision**

The current discussion of color vision would be incomplete without a brief survey of the diversity of color vision systems in nature. There are several studies looking into the color vision systems of invertebrates (Figure 1.3.4 B-D), but probably the best described ones are the fly, *Drosophila melanogaster*, the honeybee, *Apis mellifera*, the butterfly, order *Lepidoptera*, and the mantis shrimp, order *Stomatopoda*. The former is a very well described model for neuroscience, and hence the methodologies developed to explore its visual system become very useful to explore its color vision system. The fly eyes are compound eyes, where instead of a lens projecting an image of the world into a light sensitive tissue, each single compartment, called ommatidium, captures part of the visual environment directly and then relays this information to the fly optic lobe. Inside the ommatidium there are several compartments expressing a single opsin. The first six are in theory used for luminance and motion vision (R1-6). Beneath these compartments lie the compartments thought to contribute to color vision (R7 and R8), since depending on the type of ommatidium they express different subsets of opsins sensitive to different parts of the visual spectrum, including the UV range [60]. These compartments also project to the optic lobe, but skip the first layer, termed the lamina, and project directly into the second layer named the medulla. From here medulla neurons project this information into the lobula and lobula plate and then outside the optic lobe into the mushroom body and other structures [61]. It is not known how color information is processed beyond the optic lobe, but it has been shown that flies have functional color vision and can learn
color based associations \([62, 63]\). These results are compatible with findings in honeybees and butterflies, also displaying highly functional color vision systems, although described in less detail \([64–66]\).

The mantis shrimp on the other hand is well described because it expresses around twelve different types of opsins (depending on the species, Figure 1.3.4 F), and hence has the potential for a twelve-dimensional color space \([67]\). Recent research has shown that this is not the case \([68]\), since training assays showed that the animal has relatively poor color vision. It is thought that the way the different opsins are used is by vertically scanning the eye across an image, and the color information derived is based on whichever opsin activates the system the most \([69]\).

In the vertebrates, the diversity of color vision is comparable to the invertebrates. As mentioned above, fish show a tremendous range of color vision systems, all the way from non-existent to extremely rich \([70, 71]\). Most teleost fish express four types of cone opsins, and tetrachromacy has been shown in adult goldfish and zebrafish \([72, 73]\) among others. The mechanisms behind these systems are not well described, although as mentioned earlier it is theorized that fish might be doing a lot of their color processing at the level of the retina, given double opponent cells can be found in this layer already \([49]\).

Reptiles and amphibians have less well described color vision systems, although tri- and tetrachromacy are widespread as mentioned above \([75, 76]\). Reptiles also developed the presence of oil droplets in front of some of their cones. These colored pigments reduce the amount of light getting to the particular cone they mask, but more importantly, they high-pass the wavelengths impinging on that cone, generating more diversity in the input and hence potentially expanding the color discrimination abilities of the organism \([77]\).

Birds seem to have a fairly well developed color vision system, with colored oil droplets and also UV vision in several species \([78]\) (Figure 1.3.4 E). This has been shown to be relevant in the finding of mates and conspecifics \([79]\), and also in foraging \([80]\), both of which constitute very strong evolutionary drives. Early work in pidgeons showed their tri-chromacy \([81]\) and later work has showed functional color vision in several species \([82]\).
Figure 1.3.4: Photopigment spectra for a variety of species. For all the plots, the y axis is normalized absorption and the x axis is wavelength. A: Human B: Water flea *Daphnia* C: Honeybee D: Butterfly *Papilio xuthus* E: Blue-tit *Cyanistes caeruleus* F: Mantis shrimp *Neogonodactylus oerstedii*. Adapted with permission from Justin Marshall and Kentaro Arikawa. Unconventional colour vision. *Curr Biol*, 24(24):R1150–R1154, 2014. ISSN 0960-9822. doi: 10.1016/j.cub.2014.10.025
Finally, as mentioned above, mammals have relatively poor color vision systems given their nocturnal origins \([58, 83]\). Most pigments were lost through evolution and hence most mammals are dichromats. As with flies, the best described systems are the ones from established model organisms, such as primates and rodents. Primates are either trichromatic or dichromatic depending on the species. Interestingly, this also can depend on the gender of the animal. New World monkeys are generally dichromats since they only express a SWS1 pigment and a LWS one, but the latter is in the X chromosome and hence females can become trichromats via mosaic expression of two polymorphic LWS pigments via X inactivation (reviewed in Jacobs \([84]\)). This is of special interest, since there are no observed additional changes in the system wiring other than the expression of this additional opsin, arguing for an inherent capacity of the system to generate better color discrimination just by adding an extra opsin \([85]\).

Rodents are starting to be better described in terms of color vision. They have the particularity that their two opsins are expressed in a dorsal to ventral gradient in their retina. This gradient is realized by segregation of the green and UV cones in the dorsal and ventral retina, and gradient co-expression of the green and UV opsins in the cones in between \([86, 87]\). Color signals are combined by bipolar cells, which also incorporate rod signals when available to generate better color discrimination \([88]\). These signals are relayed to RGCs and eventually to V1. Interestingly, as in female New World monkeys acquiring an extra opsin via mosaic X inactivation, when a third, human opsin was inserted into the mouse genome and expressed in the eye, the mice showed functional trichromacy, also arguing for a circuit already compatible with multiple pigments \([89]\).

1.4 Zebrafish biology and neuroanatomy

The larval zebrafish has recently become a very popular model for the study of the nervous system. In the next paragraphs, the reasons for this choice will be presented and discussed, as this fish will be the focus of all the research presented in this thesis.
The zebrafish *Danio rerio* is a relatively small tropical fish belonging to the teleost family. They can be found in the rice fields and streams of India and Pakistan, but they are currently commercialized almost worldwide as an aquarium fish given their relative resilience and ease to breed [90]. In particular, a single female can lay around 300 eggs per clutch, which are fertilized externally by the male and then develop into a fertile adult in around 3 months time. Adults are sexually dimorphic, but larvae do not have a defined gender. Although in their natural environment they breed seasonally, when grown in the laboratory they can be bred bi-weekly for several months [91].

In terms of their daily cycle, their activity decays at night and the periods of this decreased activity are regarded as sleep. These decreases in activity are entrained in a circadian manner, and in the laboratory they are normally grown in a 14/10 hour day/night cycle. Most of these features are shared between the zebrafish and many other fish species, but what set them apart as a model organism was their development [92].

Zebrafish as a model organism

As prefaced above, zebrafish were first used as model organisms not for neuroscience, but for developmental biology. In the 1960’s, George Streisinger started using this animal for studying early embryonic development, since aside from the large numbers of embryos produced at a time and their fast development, zebrafish embryos are almost fully transparent and hence the stages of development can be readily observed over days [93, 94]. This feature was used in combination with forward genetic screens to identify genes relevant in the development of this model [95, 96]. This was also powered by the ease of use of morpholinos in the larval zebrafish. Morpholinos are small fragments of modified DNA that trigger degradation of the RNA molecules they basepair with, allowing for targeting of specific genes [97]. These two features helped propel zebrafish as a developmental model but also as a genetic model.

It was not until the development of calcium functional imaging and 2-photon microscopy that the zebrafish started increasing in popularity as a neuroscience model organism [98, 99](Figure
1.4.1 A). These two techniques allow for the real-time observation of brain dynamics in the intact, awake animal. Given the small size and transparency of the zebrafish (Figure 1.4.1 B), they allow for full brain imaging, which is a unique feature among all vertebrate model organisms (Figure 1.4.1 C). The other important component of this inclusion is the behavioral array of the zebrafish. Even at the early stage of development the larvae are normally imaged at (5 to 7 days post fertilization, dpf), they show a large array of complex behaviors [100], and their visual system is functional already at 4 dpf [101].

Anatomically, the brain of the larval zebrafish is around $1000 \times 600 \times 400$ um in size (Figure 1.4.1 D). It is divided into hindbrain, midbrain and forebrain as in most vertebrates [102]. As with all fish, it lacks a cerebral cortex, although analogies to cortical structures have been proposed [103, 104]. Additionally, given it is an organism in development, many of its brain structures do not have clear correspondence to the adult vertebrate brain, although accumulating evidence from recent years has begun to change that. The visual areas of this brain are of particular interest for this work, and will be discussed in more detail below.

**Visual system neuroanatomy - Retina**

In terms of their visual system, zebrafish have a layered retina like the rest of vertebrates, also with outer and inner nuclear layers, outer and inner plexiform layers and a ganglion cell layer. Moreover, the general cell types found in other vertebrate retinas are all present, namely rods, cones, horizontal cells, bipolar cells, amacrine cells and ganglion cells [101, 106, 107].

As mentioned briefly above, the larval zebrafish expresses four classes of cones, and no rods until 15 dpf [108]. The four cones are centered at 362, 410, 480 and 570 nanometers, giving the animal the potential for tetrachromatic color vision including the UV range. These cones differ in shape and position in the retina, and they form a stereotyped mosaic pattern typical of fish but absent in most other vertebrates [108–110]. All four cones have been shown to be functional at 4 dpf, with retinal spectral sensitivities that show a preference for short wavelengths [111].
Figure 1.4.1: The zebrafish larva as a model organism A: Schematic of a standard setup for stimulus presentation, behavioral monitoring and neural imaging, combining a projector, a camera under the animal and a 2-photon microscope on top. B: Picture of a 6 dpf larval zebrafish. The red box indicates the region imaged in C and D. C: Anatomical location in the brain of response clusters obtained from recording responses under OKR stimulation and separating based on phase shift between the stimulus and response. The scale bar is 50μm, Ro is rostral, C is caudal, L is left, R is right. D: Schematic of the brain regions depicted in C. Adapted with permission from Ruben Portugues, Claudia E. Feierstein, Florian Engert, and Michael B. Orger. Whole-Brain activity maps reveal stereotyped, distributed networks for visuomotor behavior. Neuron, 81(6):1328–1343, 2014. ISSN 0896-6273. doi: 10.1016/j.neuron.2014.01.019
As with other vertebrates, the cones of the zebrafish also make ribbon and flat synapses generating ON and OFF pathways in the retina respectively \(^{112}\). Hence, one can also find ON, OFF and ON-OFF cells at the RGC level \(^{99}\). The connectivity of the bipolar cells to cones and to ganglion cells is not very well defined, except for a single anatomical study that identified at least nine types of projections \(^{113}\). Aside from the connections to bipolar cells, the zebrafish also has at least three types of horizontal cells that have relatively stereotyped connectivity patterns \(^{114}\). These have been explored functionally and have been shown to have color selectivity, putatively participating in color vision or at least in the generation of color constancy, as has been proposed before in the goldfish \(^{115}, 116\). Amacrine cells were recently described physiologically and anatomically, with several types of neurons identified, although the study worked with very few cells \(^{117}\). Finally, ganglion cells have been more thoroughly characterized based on their projection targets. These comprise ten regions in the larval brain, as described by Burrill and Easter \(^{25}\) and then confirmed by other studies \(^{118}\). The regions are termed Arborization Fields (AFs, Figure 1.4.2), and go ventro-dorsally following the optic tract past the Optic Chiasm. They are numbered 1-9 in ventro-dorsal order, all the way into the Optic Tectum, which is AF10. There have been several studies exploring the functional significance of these AFs and their targets \(^{105}, 119–121\). It is known that \(~ 97\%\) of RGCs make it to AF10 and send some or none collaterals into the other AFs. The remaining cells end their projections in AF9 \(^{118}\).

**Visual system neuroanatomy - Optic Tectum**

As one of the largest areas in the zebrafish brain, the Optic Tectum is the main retinorecipient area in this animal, and it is the putative site for multisensory integration in the absence of a cortex, as will be described below.

The structure of the OT in fish can be divided in two regions. The top region contains several layers of neuropil where all the inputs and outputs interact, and the bottom layer is a densely packed cell layer, called the Stratum periventriculare (SPV). Most neurons in the OT reside in the SPV and extend their dendrites up into the neuropil layers. The top layers of the OT (Stratum opticum and Stratum fibrosum et griseum superficiale, SO and SFGS respectively) receive most inputs from the
RGCs (Figure 1.4.3). The individual RGCs innervate one and only one of the layers, although as mentioned above some of them make collateral connections into the retinal AFs. The outputs of the OT come from the lower layers (Stratum griseum centrale and Stratum album centrale, SGC and SAC respectively), though the axons from the projection neurons stem from their monopolar projection into the upper layers [30, 122].

In terms of circuitry, the RGCs synapse into tectal interneurons that can either synapse with projection neurons going into premotor areas or into other tectal interneurons. The connections between interneurons can be excitatory and inhibitory (Glutamate and GABA mainly, [124]) although there are some cholinergic cells [30]. The properties of these neurons were described early on in the adult and then more recently in the larva. Several of them are direction or orientation selective, sensitive to moving or flashing spots and also size selective [99, 125].
Figure 1.4.3: Zebrafish optic tectum layering A: Image of the larval zebrafish optic tectum expressing Synaptophysin-GCaMP3 in RGCs in the green channel. The pink represents a Dil injection into the right eye. The scale bar is 50μm, L us lateral, A is anterior. A’: Zoom into the white square in A, on the green channel. The scale bar is 20μm, SAC: Stratum album centrale, SGC: Stratum griseum centrale, SFGS: Stratum fibrosum et griseum superficiale, SO: Stratum opticum. A’’: Same as in A’ for the Dil channel. Scale bar is also 20μm. Adapted with permission from Nikolas Nikolaou, Andrew S. Lowe, Alison S. Walker, Fatima Abbas, Paul R. Hunter, Ian D. Thompson, and Martin P. Meyer. Parametric functional maps of visual inputs to the tectum. *Neuron*, 76(2):317–324, 2012. ISSN 0896-6273. doi: 10.1016/j.neuron.2012.08.040
1.5 **Zebrafish behavioral repertoire**

Given their external fertilization, the larvae of the zebrafish have been constrained by evolution to a very fast development into a highly functional and independent organism. The array of behaviors displayed at 5 dpf is a manifestation of this development.

**Non-visual behaviors**

Spontaneous swimming is comprised of discrete events called bouts, either directly forward or making a turn, and separated by periods of no mobility. These bouts make segmentation of larval behavior a much easier task given their discrete nature, and has allowed for very detailed quantification of their swimming dynamics. As shown by Dunn et al. [126], the larvae tend to chain turns in the same direction when swimming in an open arena, and will engage in active exploratory behaviors.

If the larva senses an auditory or tactile stimulus of sufficient magnitude, this will elicit a highly stereotyped, high angle fast escape turn in the order of a few milliseconds [127]. These escapes have been thoroughly explored and it is known they are driven in part by direct projections from auditory sensory areas into a giant, bilateral cell called the Mauthner that directly drives motor neurons [128].

An added sense exclusive to aquatic animals is the presence of the lateral line, an organ based on hair cells similar to the ones in the ear that is in charge of sensing changes in water flow around the animal. This organ is present even in larval zebrafish, and recent work has shown that the zebrafish utilizes it to maintain its position in a stream when it gets dragged by the flow. The use of the flow information to do this is termed rheotaxis, and in the zebrafish larva it is based on the sensing of curl around the fish and its navigation based on the flow gradient [129, 130].
Optomotor response

Aside from these advances, by far the modality that has been more heavily explored is the visual system. There is a large array of visually driven behaviors under different levels of inquiry. Perhaps the most well known is the Opto-motor response (OMR), where the larva will swim in the direction of movement of sinusoidal or square wave gratings oriented perpendicular to their direction of motion \([131, 132]\). This behavior is thought to arise from the need of the larva to maintain its position in a stream despite the current, so that if the larva starts begin dragged (visual flow goes forward) then it will attempt to cancel the motion of the visual flow by swimming forward also and remain in place. A long series of publications has been piecing apart this behavior, all the way from the sensory areas involved and into the spinal circuits in charge of actually generating swims \([120, 133, 134]\). It is now known that direction selective information is sent via the RGC axons to AF6, which then relays this information to the Pretectal area. The Pretectum then processes the information from both eyes and drives the nucleus of the MLF to generate forward swims. A separate tract of information leaves the pretectum to the hindbrain, where additional stages of processing generate turns \([121]\). The main caveat with these advances is that perturbation studies are required to check the model assumptions at every stage, but it seems the field is approaching full circuit understanding.

Phototaxis

Phototaxis is the tendency of an organism to move from areas with less light to areas with more light. This is a very widespread behavior in nature, observed even in bacteria \([135]\). In the zebrafish the behavior has been fairly well characterized. The assays utilized are either the positioning of a bright spot in an open arena \([136]\) or the projection of a split field with light and dark underneath the fish, which then follows the animal keeping the boundary in its center of mass \([137]\). The former was utilized, in combination with ablations and pharmacology, to determine that phototaxis depends on the luminance of the adapting light before the fish goes into darkness, and that the ON visual pathway gates forward swims while the OFF pathways gates turns away from darkness. The latter system was utilized to describe the stereotyped turning angle utilized when avoiding
darkness. Recently, the dorsal left habenula was implicated causally as the locus for light preference, receiving direct projections from AF4, but the connectivity past the habenula and into motor areas is still unknown [138].

**Dark and light flash response**

Although less intense than acoustic startles, quick transitions between light and dark and between dark and light can generate startle responses in the larva [139, 140]. Dark flashes generate a very stereotyped escape turn termed an O-bend, due to the shape of the animal when performing the movement. This turn shows more latency after stimulus than acoustic startles, but it still occurs within a few milliseconds. Light flashes elicit a much more delayed startle response, instantiated by a large angle turn different from the O-bend. Both types of responses are subject to habituation in the same way as acoustic startles, and are also functions of the contrast between the light intensities before and during the flash. As with phototaxis, the neural substrates of these responses are unknown.

**Prey capture**

Perhaps one of the most heavily studied behaviors in the larva, its ability to capture prey is a very complex behavior relying heavily on vision for its execution. The canonical hunting sequence for moving prey - usually live paramecia - consists on eye convergence onto the target, followed by approach to the prey, orienting towards it using a stereotyped tail movement (termed a J-turn) and finally a strike movement [141]. Different groups have identified relevant regions for this behavior. The earlier reports point to the OT as being necessary for prey capture [142], which is consistent with earlier reports of some neurons there having receptive fields tuned to small moving objects [125]. A much more detailed study [143] followed up on the tectal findings and found defined, unmixed cell response types that are preferentially activated before a hunt is initiated, most of them related to the detection of small objects. Later, AF7 was also identified to be relevant for the behavior, especially given neurons there seem to be sensitive to the size of the objects in the visual field.
The neural correlates past the visual areas are currently under scrutiny. [119].

Optokinetic reflex

Also an extremely conserved behavior, the optokinetic reflex consists of the tracking eye movements, interspersed by fast saccades, that are triggered by moving gratings shown to the animal. Portugues et al. [105] used whole brain calcium imaging to describe the general areas involved in the processing of the visual stimulus and in the generation of motor output. In particular, activity was observed at sensory, sensory-motor and motor areas in sequence, starting with the OT, the pretectum and thalamus, and the inferior olive, followed by different hindbrain areas. The activity map was extremely stereotypical across individuals, but further research into each of the involved areas will be needed to understand exactly how is the behavior generated. These findings are supported by a parallel study looking at the particular cell responses in AP6, and the analysis of their response types to support both the OKR and OMR [120].

Looming response

In the zebrafish, the looming response manifests as an escape response triggered by an object that enlarges over time in the visual field. Two groups have recently described the behavioral and neural aspects of the response. Both studies utilized expanding circles projected from below the animal and described the ideal parameters to elicit the behavior, a black circle on a white background [144, 145]. The neurons triggering the response react after a certain visual critical size is reached by the looming stimulus, starting a multimodal chain of events leading to motor output through the hindbrain.

Zebrafish color vision

The larval zebrafish has the potential for tetrachromatic color vision based on its four types of cone photoreceptors and four corresponding photopigments [106, 109, 146]. This has been shown to
be the case in adult zebrafish based on a learning assay [72]. Additionally, the four cones have been shown to be active, and the spectral sensitivity of the animal does cover the entire spectral range determined by the opsin spectra [147, 148]. Studies in larvae have been scarcer, in part because of the lack of a widespread training paradigm, which prevents the use of classical behavioral assays for color vision. Instead, the main source of information has been the use of Electroretinograms (ERGs) to assess spectral sensitivities at the level of the retina. This technique, akin to electrocardiograms and electroencephalograms, allows for assessing the response of putatively different layers of the retina. In particular, the first deflection, or a-wave, characterizes the responses of the cones, and the b-wave the responses of the bipolar cells [149]. From these analyses it was established that zebrafish larvae do have a full range spectral sensitivity, but their sensitivity for UV and blue cone targeting stimuli is much higher than for red and green, probably because the latter are not yet fully active [111]. The combined studies listed above did agree on the existence of opponent mechanisms in zebrafish color vision, with the UV and blue inputs acting both independently and additively, and red/green and green/blue only as opponent interactions [147, 148].

Despite the lack of a learning paradigm, there have been attempts at using innate behaviors for probing color vision in the larva. Two studies attempted the determination of the color preference of the larvae, but they do not take into consideration the spectra of the cones or their stimuli, and show contradicting results, pointing to place preference not being a relevant stimulus for color [150, 151]. There is also a study using the OMR response to determine directionality of turning based on color contrast, but the authors found the larvae are red-green motion color blind [152, 153], as is the case with the adult. This conspicuous lack of behavioral assessment of the larval zebrafish color discrimination was the motivation for part of the present work.

1.6 Calcium imaging

This section deals with the main method utilized in this work, namely functional calcium imaging. The section will discuss the reasoning behind using calcium as a proxy for neuronal activity, its caveats and the current state of the field.
Calcium dynamics in neural signal transmission

Calcium is a ubiquitous second messenger in most living systems, and as such is involved in several cellular processes, mostly as an intermediate in biochemical pathways. In general, calcium concentration is tightly regulated so that the cytosolic amount is extremely low, and small, local fluctuations can trigger relatively large changes with very fast kinetics [154, 155]. The common reservoirs of calcium are the ER and the extracellular space. Hence, the receptors and channels that are responsible for calcium concentration fluctuations are located in the ER membrane and the cell membrane. There are several types of channels and receptors that depend on calcium, including but not limited to voltage gated calcium channels, calcium permeable AMPA receptors and NMDA receptors among others. Additionally, metabotropic glutamate receptors can trigger calcium release from the ER [155].

The reason calcium became of special relevance for neuroscience was the discovery that neurotransmitter vesicle release depends on the action potential-dependent influx of calcium into the presynaptic terminal [1]. This influx is mediated by voltage gated calcium channels that open when an action potential reaches their vicinity. Once open, these channels allow calcium in, which starts a signalling cascade resulting in the release of neurotransmitter vesicles into the synaptic cleft. The entire process is extremely fast and can happen several times per synapse. This meant that calcium could be used as a proxy for neuronal activity, given its fluctuations can be detected reliably [1, 2, 99, 156].

2-photon microscopy in calcium imaging

Given this correlation between calcium and neural activity, several iterations of biochemical and genetic tools were (and are still) in development to capitalize on this knowledge. The tool of choice for the work presented in Chapter 3 is the genetically encoded calcium indicator (GECI) GCaMP6s [157]. This indicator was designed from the green fluorescent protein (GFP), but to make it sensitive to calcium its creators tethered two domains from calcium interacting proteins Calmodulin and Myosin light chain [158]. In the presence of calcium, these domains join together,
but in the absence of calcium they separate, and both processes are performed under relatively fast kinetics (from 200 ms to about 2 s depending on the variety of the indicator used [157]). These domains were attached to the ends of a circularly permuted version of GFP, so that its N- and C-terminus are around the chromophore, the region of the protein responsible for generating fluorescence via photon absorption. Since the environment of the chromophore has an important influence in its fluorescence, there is a very large difference in the fluorescence of the indicator in the presence versus the absence of calcium, allowing for detection of neural activity. The kinetics of the indicator are not fast enough for separation of successive single action potentials (on the order of a few ms [1]) or bursts, but their signal to noise is very high and are able to reliably detect individual action potentials in most compartments of the cell [99, 159, 160].

During development of GECIs and calcium imaging, 2-photon microscopy entered the field and became the tool of choice to be able to image these indicators [161, 162]. The principle behind 2 photon microscopy is the idea that 2 photons of a higher wavelength (lower energy) reaching the same place at approximately the same time will add their energies for absorption, resulting in emitted fluorescence as if a photon of twice the energy was absorbed [163](Figure 1.6.1 A). This results in a very tight focal spot, given the sharp decay of the probability of 2 photon coincidence with distance (Figure 1.6.1 B). This focal spot permits the acquisition of very high resolution images, since the fluorophores above and below the focus do not get excited by the laser and hence out of focus fluorescence is greatly reduced [161]. Additionally, because of the above, photobleaching is also greatly reduced, allowing for longer imaging sessions. Finally, since the wavelengths used to excite the fluorophores are higher, they penetrate tissue better, allowing for deeper imaging that in regular confocal scanning microscopy [161, 164].

All of the above advantages were combined with the inherent transparency of the zebrafish brain and the versatility of GECIs to yield an extremely powerful system for the examination of neuronal circuits at a whole brain level [105, 166].
Figure 1.6.1: 2-photon effect and its confined focal spot

A: Jablonski diagram of the 2-photon effect. When 2 photons of half the energy required to excite from particular fluorophore from the ground state to the excited state coincide, their energy is enough to excite the fluorophore and generate emission.

B: Schematic of the excitation cone of linear excitation compared to the restricted excitation focal point of 2-photon microscopes. Adapted with permission from F Helmchen and W Denk. Deep tissue two-photon microscopy. Nature methods, 2005. ISSN 1548-7091. doi: 10.1038/nmeth818

1.7 Calcium imaging data analysis

To close the introduction, this section will cover some of the common approaches to extract neural circuit information from the results of calcium imaging. It begins with a brief overview of the dimensionality reduction methods used after the pre-processing of the raw data, and then it will touch upon an array of available techniques to attempt and extract knowledge from these data.

Dimensionality reduction

Given the magnitude of the data sets generated via modern calcium imaging experiments, there has been a lot of development in the area of extracting the features from the data that carry most of the information, while reducing its effective size in memory. Since in the extreme case, in time traces every independent time point can be considered a separate dimension, ways of condensing features
to use less time points are effectively methods for reducing the dimensionality of the data [167]. The most trivial way of doing this is averaging across time points, but as can be inferred, this neglects a large amount of information in the data (in most cases at least). The next approach of choice is principal component analysis (PCA, Pearson [168]), which realigns the reference system of the data in its high dimensional space so that it is aligned with its axes of most variability. This allows for discarding all the dimensions that contain less variability, since they contribute no information. A sparse implementation of this technique is utilized in Chapter 3 [169]. Some recently popular alternatives to traditional dimensionality reduction involve visual data embedding techniques, so that under a particular algorithm the data is projected into a lower dimensional space based on the high-dimensional similarity of its components (i.e. similar elements in the high dimensional space are plotted closer together, and conversely dissimilar elements are plotted further away). This way, an algorithm prioritizing particular relationships within the data can embed it in a space of less dimensionality, an example being t-distributed stochastic neighbor embedding (t-SNE [170]). There is a large variety of dimensionality reduction methods, and the choice generally depends on the constrains of the experiment and the data analysis. For this case and all the following, parallel computing can be a powerful tool for processing time reduction if the hardware is available [171].

**Regressor analysis**

Perhaps the most basic approach to extract information from a population of traces is the use of regressors. This technique involves the design of vectors containing features of interest, and then correlating these vectors with the population of traces. This will yield a score that can be used to classify the traces under each regressor, and hence get an idea for the profile of responses in the population. The advantage of regressors is that the allow the testing of very direct hypotheses, but this also comes with a regressor design problem: Designing a regressor intrinsically constrains the analysis to what is contained in the regressor, and hence it might miss important components of the response traces if they are not considered. To circumvent this, an alternative is to use a separate, data-driven technique to derive the regressors, such as PCA for example. An implementation of the method can be seen in the work of Bianco and Engert [143], Kubo et al. [120] or Portugues et al. [105].
Clustering

A more unbiased approach to obtaining a response profile is the use of clustering methods. These belong to the family of unsupervised machine learning, since the software is designed to find patterns in the data and then group the individual traces based on these patterns. There is a large number of methods available for clustering, but they can be separated in methods that require a number of clusters as an input and methods that do not. The former allow for discrete separation of the data, since they will allocate each trace within a specific cluster, but cluster number determination is a non-trivial matter in general and this tends to be very application specific. Some examples are K-means \cite{172} and Gaussian Mixture Models \cite{18, 173}. When cluster number is not necessary, such as in hierarchical clustering, the data will be grouped based on distance, and the user needs to determine a threshold of distance to separate them in discrete groups. As with the first methods mentioned, setting this threshold is also an application-specific problem. Another caveat of clustering methods is that they can be very computationally demanding with large data sets, so recent efforts have been directed at making the methods more efficient, and in most cases some sort of dimensionality reduction is required \cite{174}.

Classifier training

The approaches discussed above have a clear connection to the data, so that individual traces are assigned to a particular cluster or regressor, and the output is a set of representative responses. An orthogonal but complementary approach is to instead probe the set of responses for the encoding of a particular stimulus. Classifiers belong to the realm of supervised learning, and allow precisely this process \cite{173}. A classifier can be trained on labeled examples from the data, so that it separates the different kinds of stimuli based on the responses they elicit. Then it can be tested on a withheld part of the data set, and its performance will be a reflection of how well the data can encode the stimuli \cite{175, 176}. This of course has a large number of caveats. These mostly derive from the fact that classifier performance will depend on how it is structured, namely a fully linear classifier might perform poorly not because the target information is not present in the data, but because it might be encoded in a non-linear fashion that is invisible to the classifier. Conversely, a classifier might
perform really well based on spurious information that is not really available to the biological system, or simply because it is supplied with enough variables to encode the stimulus from unrelated response features. Considering these caveats, the particular classifier (trees, support vector machines, logistic regression, neural networks, etc) and its parameters need to be selected carefully based on the available data, and several validation stages must be included (such as training the classifier with randomized data to establish boundaries of accuracy for example)\cite{173}.

**Output regression**

When the variations in the stimulus are in the order of variations in the data, or when two related regions have been recorded, another option is to ask whether the responses on one region (or the stimulus itself) can be in any way transformed to obtain the responses at the second region. This is done via the process of regression, and if successful it offers an explicit model from one region to the next \cite{173}. As with classifiers, this only indicates that the information to perform this calculation is there, but not that the neural circuit is utilizing it in that way. Conversely, an unsuccessful regression often means that the model selected was incorrect, not that the information for the computation under question is lost between the two target regions. Regardless, regression is an very powerful approach to test direct hypothesis of the way in which inputs are combined (e.g. linearly vs non-linearly) and if properly controlled it can shorten the path to a model of the neural circuit.

**Input decorrelation**

Finally, a method utilized generally for sensory system analysis is to quantify the decorrelation of the inputs to a system, as a result of the computations performed on that system \cite{177}. A commonality in sensory systems is that they receive input from a large number of sensory receptors with different tuning, and based on those profiles of activation the circuit needs to be able to single out the different stimuli, even the ones that elicit similar responses from the receptors. One way to achieve this is demixing by decorrelating the inputs, so that every pattern ends up being unique and well separated from the others in a high dimensional space \cite{178, 179}. While the conclusions that
can be derived from this kind of analysis are limited, it is generally an indicator of neural processing, since if the information is not being used at a particular stage and it is only relayed then this decorrelation will not be observed. Any conclusion of this type needs validation through other, more direct methods, but it helps in guiding experiments and it is generally easy to compute.
2

Phototaxis and avoidance as a function of wavelength

2.1 Background

The ability to detect and use colors is ubiquitous in nature, and is an important element of feature detection in visual scenes \([180]\). Color vision begins with the wavelength selective absorption of light by the eye’s photoreceptors. Activation of these cells triggers a signaling cascade that propagates the transduced light information into several layers of processing, both at the retinal level and beyond \([7]\). The detection and perception of color has been studied at many of these
levels and in a variety of organisms, but we still do not understand many of the basic principles governing color vision [32].

Larval zebrafish have recently emerged as a model organism for the study of vision given their wide array of visually guided behaviors, such as the Opto-Motor Response (OMR, swimming in the direction of full field optic flow), Opto-Kinetic Reflex (OKR, eye movements for tracking moving objects), phototaxis (swimming from dark to light areas) and prey capture among others [181]. Their relevance in color vision research comes from their tetrachromatic retina, since aside from rods they have four types of cones that are morphologically distinct (Raymond et al., 1993). These cones are selective for four different wavelength ranges with varying degrees of overlap and functionally develop within the first three days post-fertilization [101, 182, 183]. Three of these cones have their center of absorption in the visible light range, namely at 570 (L cone), 480 (M cone) and 415 nm (S cone) and, interestingly, their fourth cone is UV sensitive, with an absorption spectrum centered at 362 nm (Figure 2.2.1B) [109, 184].

Given this potential for a rich color vision there have been a number of studies tackling the subject in adult fish. Three independent studies used training paradigms to evaluate color preferences of the fish, but found contradictory results and there was no control for stimulus luminance [150, 151, 183]. Risner et al. [72] performed appetitive learning-based studies to test the wavelength discrimination capabilities of adult zebrafish, and to measure the behavioral spectral sensitivity function of the animal. By contrast, the effects of color stimuli on the behavior of larval zebrafish is relatively undescribed, despite a plethora of anatomical and electrophysiological studies that examine color processing circuitry in the larval retina, looking at connectivity [183, 186], functional properties [109–111] and ontogeny [182, 187]. The only behavioral study targeting color perception in the larva is centered on the OMR behavior, and it found that zebrafish larvae are red-green color blind to motion [152]. Hence in this chapter we contribute additional knowledge on larval zebrafish innate color sensing by measuring the behavioral responses of larvae to stimuli spanning the absorption range of their cones, and devoid of motion components. In particular, we will be relying on phototaxis, the tendency of the fish to move from areas of less light intensity to more light intensity. This behavior is not as robust as the OMR response, it has a dependency on the adaptation light level prior to the behavioral trial [136] and it has a short memory-like component
[188], but it does not involve a motion component in the visual field of the fish. Therefore it is ideal to test color stimuli in light of the findings by Orger and Baier [152] outlined above. The neural circuitry required for this behavior has not been fully elucidated, but Burgess et al. [136] proposed a mechanism in which the ON retinal pathway drives forward swims towards light sources while the OFF pathway drives turns against darkness. Given the latter work was performed using a relatively slow paradigm, Huang et al. [137] developed a new phototaxis paradigm where a split field is projected below the fish, with one stimulus at each side. This field tracks the animal in real time using a feedback algorithm to update the projection position based on the location of the animal. Hence, it allows for very high temporal resolution in evaluating stimulus selection since each turn is a choice between the stimuli on one side versus the other. This will be the main methodology for this study.

Although this chapter examines the majority of the spectral range of zebrafish vision, the UV region of this spectrum is of particular interest given the diversity of ways UV vision has been utilized across the animal kingdom, and its unexplored usages by this model organism. Over the last 150 years, ultraviolet vision has been discovered and studied in many species, ranging from invertebrates such as ants [189] and bees [190] to fish [70], birds [191] and mammals [192]. These studies found that UV signals are involved in many different behaviors, including mate choice, navigation and foraging. In fishes, there have been descriptions of mate selection via UV signals [193], UV-based foraging [194] and UV avoidance [195, 196]. In zebrafish, Nava et al. [197] probed the responses of adult zebrafish to UV light in a split tank assay and found an increase in escape responses, but the responses of larvae, which develop UV cones early and have a large peak in the UV region of their spectral sensitivity [111], remain largely undescribed. Given the transparent nature of this animal and the presence of active UV cones in its retina, we hypothesized that larvae should react aversively to UV light sources.

Taking all of the above into account, we set out to describe the larval zebrafish’s innate responses to visible (defined as having a wavelength > 400 nm for the rest of the chapter) and UV light using an existing phototaxis paradigm in closed-loop.
2.2 Results

Construction of a visual stimulation instrument for free swimming larval zebrafish

In this assay a stimulus is projected below the fish on a closed loop so that it stays centered on and aligned to the animal. The stimulus shows a left/right divided field, prompting the fish to choose between the two sides whenever it turns (Figure 2.2.1 A). For this chapter we modified a projector to show stimuli centered on the fish’s L, M and S cone’s absorption spectra while still partially overlapping with the UV cone (LEDs with emission spectra centered at 606, 463 and 397 nm, Figure 2.2.1 C). Although our 3-channel projector is not capable of isolating stimulation to a particular cone type, it is still highly selective and manages to activate all four cones to different degrees as shown in Figure 2.2.1 D (centering activation on the L, M and S + UV cones respectively). Hence from here on out when referring to the red, blue or UV stimulus we will be referring to the relative excitations shown in Figure 2.2.1 D or their linear sum if using a combined color stimulus (detailed cone excitations can be seen in Figure 4.3.1 A).

Zebrafish larvae innately swim towards visible light and away from ultraviolet light

When the fish (TLAB strain) was presented with varying levels of each LED on one side and darkness on the other it swam preferentially in the lit direction for the red and blue stimuli (what we will define as phototaxis from here on), but it swam weakly towards darkness when shown the UV stimulus as can be seen in Figure 2.2.1 E (values shown are averaged preference indices ± s.e.m.). Both of these responses were observed in an intensity dependent manner across 19 fish. We also looked at the stimulus effects on swimming parameters. In particular, we looked at the features of every individual movement event of the animal (termed “bout”). As shown in Figure 2.2.1 F-G, there was no change in the total displacement per bout or in the duration of the bouts, meaning the preference change we observe is due mainly to turn direction without influences from other swimming parameters (shown in Figure 4.3.1 B). This is the first demonstration of an innate mo-
Figure 2.2.1: Zebrafish larvae perform phototaxis and UV avoidance in a closed loop paradigm A: Closed loop apparatus used in this chapter. The fish is monitored in real time at 100 fps using a camera. The larva swims freely in a petri dish arena illuminated with infrared light. As the animal swims its movements are tracked in real time and stimuli are projected from the bottom, aligned to the animal. After each experiment the larval movement is segmented into individual turn events that are condensed into a preference index reflecting the stimulus choice of the animal. B: Modelled absorption spectra of the larval zebrafish cone pigments, including the beta band of absorption. C: Emission spectra of the LEDs used to project stimuli in this chapter. D: Theoretical relative excitation each LED causes in each cone. The colors correspond to the LED colors in C. As mentioned, all cones are excited to an extent. E(i-iii): Larval turning direction preference indices for UV, blue and red stimuli against darkness. The stimuli are listed at the left side of each plot and preference index is expressed as the length of the horizontal bars (bars are mean±s.e.m., N=19 larvae from different clutches. Stars represent p-value<0.05 in a paired bootstrap test comparing the lowest power level to the others). F-G: average total displacement per bout (F) and duration of each bout (G) for each of the stimuli used above. The wavelength composition of the stimulus does not seem to have an effect on swimming parameters as shown (points are mean±s.e.m., fish are same as above).
response to UV light in the larval zebrafish.

Phototaxis and UV avoidance interact to determine turning direction

We wanted to ask whether the relationship between UV avoidance and phototaxis can be revealed by the combination of the stimuli involved in each of the behaviors. First we asked whether UV avoidance could act in cooperation with phototaxis to drive the TLAB animals away from UV and towards visible stimuli, hence amplifying the turning preference. We presented the animal with increasing levels of the UV stimulus on one side (max at 2.9 μW cm⁻²) and a constant, highly attractive level of the visible stimulus on the other (max at 5.4 μW cm⁻², Figure 2.2.2 A(i-iii)). At the light intensities used the response did not behave as cooperation between avoidance against one side and phototaxis towards the other. In particular, the fish failed to show any modulation in their response with increasing levels of UV light.

Given these results we asked the opposite question, namely, how does the fish respond when UV avoidance and phototaxis are pitted against each other? To achieve this, we designed a stimulus featuring darkness on one side of the fish and a mixture of the UV and visible stimuli on the other. We held the visible stimulus constant and increased the intensity of the UV stimulus as above. As shown in Figure 2.2.2 B(i-iii) the preference of the animal for the light side decreased as a function of the intensity of the UV stimulus. This was observed across the different visible light stimuli and with different slopes showing that UV avoidance can compete against phototaxis. Despite the clear effects in turning preference, there was no change in the fish swimming parameters as a function of stimulus (Figure 4.3.2).

Up to this point we have shown that under the conditions in this study the responses to UV and visible stimuli can interfere destructively but not constructively.
Figure 2.2.2 (following page): UV light is avoided in a manner indistinguishable from darkness and this avoidance competes with phototaxis A(i-iii): Preference shown by the larvae for varying levels of UV stimuli vs red, blue or both red and blue stimuli. B(i-iii): Preference of the larvae for darkness vs red, blue or red and blue stimuli with increasing levels of the UV stimulus. (bars are mean±s.e.m., N=21 for the A panels, N=20 for the B panels, both with fish from several clutches. Stars represent a p-value<0.05 in a paired bootstrap test comparing the lowest power value to the others).
UV avoidance is mediated by functional UV cones in the retina

Zebrafish have an extremely rich array of non-visual opsins, expressed in a number of brain regions and also outside the brain [198, 199]. Some of these opsins - such as melanopsin [200] and UV opsin itself [12] - have been shown to be behaviorally relevant. Hence, we asked whether the observed UV avoidance was mainly visually driven or not, especially given the results from Fernandes et al. [201] on a phototaxis behavior mediated by these opsins. To this end we performed phototaxis experiments in chokh/- mutants, which lack eyes due to a mutated homeobox protein [202], and we compared these to their wild type siblings. As shown in Figure 2.2.3 A(i-iii) the mutant fish show an extremely erratic response while their wild type siblings exhibit normal phototaxis. To our surprise some preference indices in the mutant fish were fairly high, but this is probably due to the lower bout frequency in this group, and is confirmed by the lack of a trend for any of the stimuli in the series. Aside from the bout frequency, all the other bout parameters are very similar between the mutant and wild type animals (Figure 4.3.3 A).

Given our behavioral results and the above experiments with the chokh mutant, we hypothesized that the UV cone in the eye must have a role in the response. To test this idea we used a transgenic animal with inactive UV cones. In this transgenic, Yoshimatsu et al. [185] inserted a cassette containing the sws1 promoter (normally driving expression of the UV opsin [203, 204]), followed by a fusion of Tetanus toxin and the Cyan Fluorescent Protein (CFP). Expression of this fusion renders the UV cones inactive and also allows for screening of the individuals with successful transgenesis [185]. When the transgenic fish were tested in our paradigm, and compared with their wild type siblings, we observed that the responses to red and blue stimuli were similar between groups but the responses to UV stimuli were abolished in the transgenic, as depicted in Figure 2.2.3 B(i-iii) (other swimming parameters remain constant, as shown in Figure 4.3.3 B). Finally, the response to UV stimuli seems to have an inverse sign, although this was found to be not significant.

In summary this evidence points to UV avoidance as a visually guided behavior that depends on the presence of functional UV cones.
Figure 2.2.3: UV avoidance is mediated by a visual process, and it depends on the presence of functional UV cones A(i-iii): Preferences of eyeless chokh mutant fish and their wildtype siblings for the UV, blue and red stimuli versus darkness. White bars show the wild type animal results and black bars the blind mutant results. (N=18). B(i-iii): Preferences of ssw1::TeNT-CFP transgenic fish with inactive UV cones versus their wildtype siblings. The stimuli consisted of darkness versus varying intensities of the UV, blue or red stimuli as in part A and Figure 1. The white bars show the wild type fish results and the black bars show the transgenic animal results (N=8, bars in both experiments are mean±s.e.m. with animals from different clutches. Stars represent a p-value<0.05 in an unpaired t-test comparing the two conditions).
Avoidance of the UV stimuli, and preference for the visible stimuli, are also affected by stimulus contrast

Visual systems tend to focus on differences rather than absolute levels [205, 206], so we attempted to measure the effect of stimulus contrast, rather than absolute intensity, on phototaxis. For this, we devised a modified paradigm to cover a range of different contrasts as a function of the turning behavior of the animal. In this experiment, the TLAB larvae are also presented with a split field stimulus, but if the fish turns towards the lighter (higher irradiance) side of the stimulus, the contrast between the two sides is reduced (by increasing the intensity of the darker side (lower irradiance) and reducing the intensity of the lighter side). If the fish swims towards the darker side then the contrast is increased (see Figure 2.2.4 A). We probed the fish responses to the red, blue and UV stimuli in this paradigm and the results can be observed in Figure 2.2.4 B. As shown, the animal successfully decreased the contrast during the red and blue stimuli by swimming towards the lighter side, but instead managed to keep the contrast high during the UV stimulus, which can only be achieved by swimming mainly towards the darker side while avoiding the lighter side. As shown in Figure 2.2.4 C(i-vi) we also observed distinct differences in a number of swimming parameters as a function of contrast. In particular, for bout frequency, bout duration, bout peak speed

Figure 2.2.4 (following page): Contrast variation but not full field stimulation have wavelength-dependent effects on swimming parameters A: schematic of the modified paradigm used. Briefly the animal is presented with a split field stimulus as in the figures above, but now the contrast between the two sides decreases if the fish turns towards the lighter side (higher irradiance) and increases if the turn is towards the darker side (lower irradiance). B: contrast of each stimulus (UV, blue and red, colored as before) over time, averaged over two trials per experiment and 14 TLAB fish (the lines are mean±s.e.m.). The units are the logarithm of the total cone excitation difference between the two sides. C(i-vi): show the modulation of swimming parameters by contrast. Although preference index seems to be relevant at higher contrasts, other parameters like turn angle, peak speed and bout duration show contrast and stimulus dependency. D-G: When the animal was presented with full field color stimuli, swimming parameters did not show stimulus dependency, although there was a significant light intensity dependence on the blue channel, with increased activity at higher light levels (points are mean±s.e.m., N=23).
and cumulative turning angle the fish seemed to show a linear, very gradual response to contrast under the red and blue stimuli but this turned into a much steeper response under the UV stimulus.

**Full field stimulation fails to elicit wavelength-specific responses**

An unanswered question from the experiments above is whether there are any discernible effects from a full field stimulus instead of a split field, since this kind of modulation will play a role in the interpretation of our preference index data. As expected, there was no turning angle preference observed in this assay (as shown in Figure 4.3.4). Figure 2.2.4 (D-G) indicates there is weak light intensity-dependent modulation in bout duration, peak speed, latency to peak speed and distance travelled. More importantly, there was no observed modulation that depended clearly on the wavelength composition of the stimulus, which is consistent with the observations from the same swimming parameters for the split field stimuli as shown in Figure 2.2.1 and in the Supplementary Information.

2.3 **Discussion**

We have shown that zebrafish larvae swim towards visible light stimuli and away from UV stimuli in a closed loop split-field paradigm. This preference is expressed mainly via turning direction with no discernible changes in other swimming parameters as a function of the stimulus. The UV avoidance behavior interacts negatively but not cooperatively with phototaxis, and relies on the visual system, and the UV cone in particular, to function. Finally, phototaxis to visible stimuli seems to be a shallow function of contrast while UV avoidance shows a sharp change.

**Functional relevance of UV light detection**

UV vision is present across the animal kingdom [53, 70, 207], serving a wide range of roles such as food and mate selection. Fish have been shown to use UV light inputs in a variety of ways, includ-
ing detection of UV pigmentation for recognition of conspecifics, enhanced foraging and deployment of protective pigments \cite{208,209}. In zebrafish it has been posed that UV detection might be used to avoid photodamage by mediating the deployment of pigment granules called melanosomes \cite{210}. This makes ontological sense since larval zebrafish have been shown to be particularly prone to damage from UV radiation due to their transparency \cite{211} and are generally sensitive to low wavelength illumination \cite{212}. The behavior we describe in this chapter is consistent with this hypothesis as it allows the animal to remove itself from regions of high UV radiation. Notably, this evidence still does not explain the positioning of the UV cones in a potentially image forming role, pointing to additional, undescribed functions that will require further investigation.

**INTERACTION BETWEEN UV AVOIDANCE AND PHOTOTAXIS**

In our study, phototaxis and UV avoidance occur, and interact, competitively rather than cooperatively. One hypothesis explaining this one-sided interference stems from the ecology of the zebrafish: larvae live at low depth with varying levels of turbidity \cite{91}, which potentially exposes them to high levels of UV irradiation \cite{213}. At the same time, the larvae rely heavily on visual input to find food and avoid predators. Therefore, a balance needs to exist between avoiding UV irradiation yet remaining in areas with enough light for their visual system. This would explain why, when avoiding UV or darkness, the response is to approach a UV-free source of light, but when the light source has UV radiation then the preference decreases as a function of the intensity of the UV component. This also answers why UV avoidance is so weak when presented on its own against darkness. However, this weak avoidance could alternatively be explained by some residual activation of circuits for phototaxis during presentation of our UV stimuli, which, in addition to stimulating UV cones, drive the S (415 nm) and M (480 nm) cones (see Figure 2.2.1 D). This would not be observed with a lower wavelength (365 nm) source like the one used in Nava et al. \cite{197} to elicit escapes in adults, since the main cone being stimulated would only be the UV cone. Along these lines, the weak avoidance of UV toward darkness may also stem from the power output of our light source, which may not maximally activate the UV cones. In this case, a more powerful source might be able to generate a stronger avoidance response during presentation of UV light. Yet another alternative is that the larvae use UV detection for polarization vision, explaining the
weak responses by the lack of polarization in our stimuli. Polarization has been described as a visual modality in other aquatic animals [214] but there are no reports of the presence or absence of this capability in zebrafish. Finally, it should be noted that in this setup we only consider possible lateral responses to UV light, and neglect any potential swims toward or away from the surface of the water. Given the increased scattering of UV light relative to other wavelengths in deeper water [213], the presence of UV light may spur larvae to move towards lower depths. This is a possibility that cannot be assessed with our paradigm but that could drive future experiments in 3D arenas.

UV and color vision circuitry

Our results with the tetanus toxin-expressing animals point to a retinofugal pathway including UV cone signals contributing to the UV avoidance behavior. Studies by Saszik et al. [111] and Bilotta et al. [215] measured the responses elicited by UV illumination in the electroretinogram (ERG) of the larval zebrafish. They report that the “a” wave component of the ERG in response to UV stimuli is distinct from the response obtained from stimulating with higher wavelengths. This difference does not originate at the photoreceptor level, suggesting the transduction of UV information into the rest of the zebrafish brain. From the perspective of connectivity, Li et al. [113] report that there are likely no cone type-exclusive bipolar cells in the adult retina, and hence it is also unlikely there are cone-exclusive retinal ganglion cells sending these signals out of the retina. This is consistent with a model where UV radiation is a very salient stimulus for the animal, but is not detected in isolation. Instead, and under the context of this behavior, it is probably represented in the most relevant way given lighting in the wild, namely as a negative component of the overall light input as explained above. Very little is currently known about color signal processing in the larval zebrafish beyond the retina, making the actual neural location and basis for the computation of turn direction from the chromatic inputs in the brain open for speculation. Candidate regions include the retina itself, the arborization fields of the optic tract, the optic tectum and downstream, undescribed areas.

The next step in deciphering this circuit will require the use of techniques to identify the regions and cell types involved, past the information that can be gained through behavior. Given the advances made during the last decade in the field of deep brain 2 photon calcium imaging in
zebrafish larva [166, 181, 216], this seems like the ideal avenue to take for furthering knowledge in this system and should spearhead future studies in the subject of UV and color vision.

2.4 Methods

Fish rearing

5-7 days post fertilization (dpf) male and female zebrafish larvae (Danio rerio, Hamilton, 1822) were bred in a 14/10 hours light/dark cycle at 28°C in 10 cm dishes. The strains used were TLAB (wild-type strain), chokh/- (strain that doesn’t develop eyes if homozygous for the mutation at this gene) and Tg(sws1:CFP-TeNT) (strain expressing tetanus toxin fused to CFP in the UV cones only). All animal protocols were in accordance with NIH guidelines and the Harvard University IACUC.

Closed loop phototaxis assay

Zebrafish swam in a 10 cm petri dish while being tracked at 100 frames per second (fps) by a camera positioned above (AVT Pike F-032B, Allied Vision Technologies, Exton, PA, USA). The camera had an infrared filter mounted to avoid interference from the visible light stimuli. Visual stimuli were presented from below by a modified DLP projector (Lightcrafter Evaluation Module, Texas Instruments, Dallas, TX, USA) with LEDs centered at 606, 463 and 397 nm to match the red, green and blue cones of the fish (Mouser Electronics, Mansfield, TX, USA). The projection is coupled to the IR illumination path by a long pass mirror (Edmund Optics, Barrington, NJ, USA). The tracking itself was performed via custom software developed in LabVIEW (National Instruments, Woburn, MA, USA). Briefly, each image is background subtracted, thresholded and then particles of a determined size are isolated and their coordinates and heading angle are recorded. Using this information the LabVIEW software also synthesized the stimuli to be shown to the fish. These stimuli consisted of a split field centered on the animal and aligned to its heading direction at every frame, prompting a choice between the sides at every turn event.
Calculation and measurement of spectra

Spectra from the zebrafish cones were calculated based on the methods and data collection described in Cameron [110]. Briefly, the calculation is based on the model developed in Govardovskii et al. [184] for A1 based pigments (including both the alpha and beta bands) and the data compiled by Cameron [110]. The LED spectra were acquired using a CCS200 UV-Vis spectrometer, and their power was measured using a PM100D power meter with an S130VC sensor (all three from Thorlabs Inc., Newton, NJ, USA). Relative cone excitations were calculated by measuring the current sensed by the power meter sensor, weighting the normalized LED spectrum by it and then using the conversion of Watts/Amperees for each wavelength provided by the sensor manufacturer. Finally this power input was multiplied by the cone spectra and integrated to generate a relative excitation value.

Trajectory processing

The fish trajectories and heading angles were smoothed and then the position trace was segmented based on stretches of continuous velocity. These events were used to find the heading angle of the fish during the inter-event period, which allowed for calculation of the turning angle between events. These angles were used for calculation of preference indices per trial by thresholding the ones that represent turns (as opposed to bouts) and applying the following formula:

\[
\text{Preference Index} = \frac{S_1 - S_2}{S_1 + S_2}
\]  

These indices were then averaged across stimuli per fish, and final values were obtained by averaging across fish. Computations for this and subsequent analysis were performed in MATLAB (Mathworks, Natick, MA, USA). The custom software written can be obtained by contacting the corresponding author.
Statistics and Error analysis

Sample size (N) for every experiment in this study is defined as single larvae. Animals were pre-screened for phototaxis to a gray stimulus and only excluded if they failed to show this behavior (pre-established criterion). Standard errors were determined by performing a bootstrap with the preference indices from each fish and 1000 iterations using the mean of these indices. The same process was performed comparing the differences between the preference indices of the stimuli of interest to test for significance at a critical value of 0.05 (two-tailed). We chose to use bootstrapping since there is no indication the data will be normally distributed, and hence opted for this non-parametric technique.

Variable contrast experiments

The paradigm used in the rest of the chapter was modified to change contrast as a function of the fish turning direction. In particular, the animal is presented with a split field stimulus in a closed loop fashion. If the animal turns towards the light side of the stimulus the contrast between the two sides is decreased. If in turn the animal swims towards the dark side then the contrast of the stimulus is increased. For determining the contrast change there was an angle change threshold of 10 degrees within a sliding window of 1 second. At each threshold crossing the intensity of one side was increased by about $\sim 5 nW * cm^{-2}$ while the intensity of the other side was decreased by the same amount.
3.1 Background

The relevance of color vision in our capacity to distinguish the various components of a scene is often overlooked. This relevance originates from the fact that differences in luminance between parts of the image may not be sufficient to separate objects from the background, or identify their boundaries [39]. Hence, via evolution, diverse organisms developed visual systems that allowed them to extract information not only from the amount of light received by their eyes but also from the wavelength of this light [7, 58].

When looking at the natural world, we see that color vision is very widespread in the animal
kingdom [59, 74], and it fulfills several essential roles, such as discerning which fruit is ripe for consumption, which surrounding animals are conspecifics of the right gender, or where is a predator camouflaging itself. Our understanding of how these systems work varies depending on the species [74]. Smaller model organisms offer more access to the visual system, but often times their visual systems are functionally and structurally very different from vertebrate visual systems such as our own [7, 217]. Larger organisms such as primates have color vision almost identical to ours, but access to the different regions of the brain involved is very limited [32, 218]. The field of psychophysics has allowed scientists to gain additional insight into color vision [219], both from model organisms and from humans, but the details of the circuitry underlying these phenomena remains mostly hidden.

The larval zebrafish offers a unique compromise between accessibility and being a vertebrate. At this developmental stage they are almost fully transparent, allowing for optical access to most of their brain[91]. This is combined with a well-developed toolbox of genetic techniques that allow expression of a desired marker in defined cell types and regions [99]. The field of 2 photon functional calcium imaging takes full advantage of the aforementioned features, allowing for real time imaging of calcium transients anywhere in the brain and from well-defined target cell types[105, 166], all while taking advantage of the latest generation of calcium indicators. These indicators show high dynamic range, high sensitivity and fast kinetics, a prime example being the GCaMP6 family [157].

The visual system in this organism offers several convenient features, such as a cone-based retina, given rods are not active until two weeks of age [108]; a layered retinal structure like most vertebrates, including humans[182]; and a large amount of accumulated knowledge about its visual system. In particular, there is extensive work done on motion perception [120, 121], responses to looming stimuli [144, 145], detection and capture of prey [119, 141–143], and luminance perception [136, 139], both from a neural and behavioral perspective.

Additionally, the larval zebrafish offers several advantages for the study of color vision in particular. It possesses four types of cone photoreceptors in its retina[108, 109], each expressing an opsin protein sensitive to a different spectrum of light, which potentially allow it to perceive color in a tetrachromatic space[58, 73, 74]. In comparison, humans and Old World primates are trichro-
matic [83], but all the standard vertebrate model organisms aside from the zebrafish are dichromatic (such as rodents, cats and rabbits) [59]. Also several of these organisms are mostly nocturnal and hence have rod based retinas, adapted for low light conditions [59].

The tetrachromacy of the zebrafish has been convincingly shown behaviorally in adults [72], and there is extensive literature on the subject based on its close relative the goldfish [15, 73]. In terms of the larva, there is less work done given the paucity of consistent and generalizable training paradigms. To date there have only been studies assessing the innate color preferences of the larvae, with mixed and sometimes contradictory results [150, 151], although there is evidence that both the adults and the larvae are very sensitive to ultraviolet light [197]. Additionally there is one publication reporting the larvae are red-green motion color blind, as humans are too [47, 152]. At a circuit level, there is previous work measuring the spectral sensitivity at the level of the retina in the larva [111]. It was observed that there is a bias in sensitivity to the short wavelengths, in particular the UV cone signals, although all four cone types were active and contributing to the retinal output. This imbalance was observed to level off in the adult, both at the retinal and OT levels [72, 220]. Also at the retinal level, it was observed that horizontal cells show differential cone type spectral sensitivities, supporting the possibility of color computations [115]. Finally, at the cellular level, there are reports of the cone types connected to the different horizontal [114] and bipolar [113] cells in the retina, which in combination offer putative, cone type selective input patterns for the retinal ganglion cells (RGCs) at the end of the retinal pathway. There is currently no further evidence of how color information is processed at the RGC or subsequent levels in the larva.

In this work, we will be focusing on the latter stage. Previous evidence has identified RGCs, and their targets in the larval brain, as the site for many essential visual computations directly linked to behavioral generation [123, 125, 221]. As described before, RGCs project out of the eye into ten different areas in the zebrafish brain [25]. The most relevant of such areas is the Optic Tectum (OT), receiving an expected 97% of the RGC projections, given the connections to other areas occur via axonal branching events [118]. Hence we will be focusing our imaging efforts on the axonal terminals of RGCs at the OT and also on the cell bodies of the OT themselves, giving us a view of both ends of this critical synapse.

To effectively sample the tetrachromatic space theoretically available to the animal [74], we will
be utilizing a custom built four channel projector capable of spanning the visual range of the zebrafish cones and additionally of stimulating the cones in at least four color directions. This is possible due to the principle of univariance, which dictates that the activation of each cone will depend both on the wavelength of light used and the amount of this light. This means the output of the cone will only be a scalar quantity that has neither the pure wavelength or intensity information, but a combination of both \[36\]. Hence, single cones are effectively color blind, and color vision systems can only function based on the combination of the signals of cones sensitive to different wavelengths. This is convenient since instead of activating the system with every possible wavelength, we can use four independent channels and generate every possible color sensation in the animal by modifying the ratios in which each cone is activated \[37\].

Given the target region and the methods for stimulating and sampling it, we will show that color information is available at the two locations surveyed, and more importantly, we show through several metrics that there are signs of color processing between these two stages, paving the way for color vision research in the larval zebrafish.

3.2 Results

2 Photon Calcium Imaging of RGC and OT Responses to Color

To answer the question of distribution of color information in the larval zebrafish brain, we constructed a 2 photon microscope compatible with the presentation of arbitrary color stimuli. This was done by controlling the timing of the scanning of the microscope with the presentation of the stimuli, so they are temporally separated. This is complemented by a gateable PMT that turns off during stimulus presentation, thereby avoiding damage or saturation to the unit. The stimuli were presented with a custom-built four channel projector tailored to the spectral range of the larval zebrafish cones (see Figure 3.2.1 B). A diagram of the instrument used can be seen in Figure 3.2.1 A.
Figure 3.2.1  **Experimental setup and target regions A:** Diagram of the imaging and stimulation setup. Briefly, a 2 photon laser is steered via galvanometric mirrors through the objective and into the sample. Fluorescence is collected by a gateable PMT via a dichroic mirror. In the meantime, visual stimulation is delivered from below using a four channel projection setup and a dichroic mirror. The stimulation and collection are synchronized so that there is stimulation only during the backscan period, during which the PMT is gated off. **B:** *(Top)* Modeled absorption spectra of the zebrafish cones, after the template in [184]. *(Bottom)* Measured absorption spectra of the four channel stimulation setup (each LED is colored differently). **C:** Diagram of the stimuli used in the study. The top panels indicate the spatial structure of the stimulus and the bottom panels the temporal structure. Only one LED was used in any given stimulus, hence the single line in the temporal structure. The full field stimulus was only shown for the four LED experiment and the remaining three stimuli were shown during the two LED/spatio-temporal experiment. **D:** Regions targeted during the study. *(Center)* Widefield fluorescence image of a 6 dpf Tg(ath5:Synaptophysin-GCaMP6s,isl3:Synaptophysin-GCaMP6s) animal. *(Left)* Magnified maximum intensity projection of a 2 photon stack through the left tectum of the animal. The location in the brain of the fish is shown with red lines. *(Right)* Maximum intensity projection of the OT area of a 6 dpf Tg(HuC:H2B-GCaMP6s) animal. For reference, the anatomical location of this region is also indicated in the center figure, with the blue lines.
Since we are interested in the distribution and processing of color information outside of the eye, we targeted our experiments to the Retinal Ganglion Cell layer and to the cells in the Optic Tectum of the animal. In particular, we expressed the calcium indicator GCaMP6s in the axonal terminal of RGCs by fusing it to Synaptophysin \[^{159}\]. In a different set of animals we expressed the indicator in the nuclei of most neurons in the zebrafish brain \[^{160}\], and restricted our imaging to the Optic Tectum. Both regions can be seen in Figure 3.2.1 D.

**Cone type weight distributions broaden from RGCs to Tectum**

The first question we wanted to answer is the relative contribution of each cone type to each one of the visual units identified in the RGC and OT regions. To probe these contributions, we used a set of four stimuli consisting of 0.25 Hz full field intensity oscillations, one for each one of the LEDs in the projector (Figure 3.2.1 C).

Given the temporal wavelength of the stimulation was known, we extracted the Fourier power of each unit and used these values as the expected response for the stimuli. Assuming linearity in the summation of cone contributions as the simplest model, we used these responses to solve for the cone “weights” at each trace (see Equation 3.1 in Methods section). Briefly, the assumption was that the response for each unit was determined by a linear summation of the contribution of each cone type and LED interaction (a 4x4 matrix), weighted by a constant value specific to each cone type and unit. Since only one LED was on at a time, the resulting matrices per stimulus could be reduced to single equations with only the weights as the unknowns. Four stimuli yielded four equations and hence a solvable system. The resulting distributions of weights for each cone type across the population are shown in Figure 3.2.2 A. The RGC and OT populations are overlaid on top of each other. The OT distributions are wider than the RGC distributions for every cone type. Also of note is the order of magnitude of the responses, since the Red and Green weights are on a similar range but the Blue weights are mostly negative and the UV weights are an order of magnitude higher. This can be seen more clearly in Figure 3.2.3 C, showing the average of each histogram.
and its variability.

The above analysis considers the cone weights individually, but it is also of interest to see if there are common trends in the relationship between the different weights for each unit. These are shown in Figure 3.2.2 B. As can be observed, while the Red weights seem weakly uncorrelated with the others, the Green, Blue and UV weights are more strongly correlated with each other. Interestingly, the Blue weights are mostly in the negative range and additionally are negatively correlated with the rest. When comparing RGC vs OT data, it is also apparent that the correlations seem to decrease in the latter. This is an indirect indication of neural processing and it will be addressed more directly below. To ensure the correlation structure is dependent on the features of each unit, we also shuffled the cone weights across units. This keeps the population of values for each cone type constant, but destroys any structure contained within each unit. This resulted in almost no correlation left between the cone weights, arguing they are not just due to population level biases

Figure 3.2.2 (following page): Zebrafish show a UV dominated spectral sensitivity, with clear differences between RGC and OT responses A: Distributions of cone weights for each one of the cone types. Each panel is a histogram of the calculated cone weights for a different cone type, for each one of the traces recorded. The x axis indicates magnitude and the y axis is count in logarithmic scale. Note that the x axes in different plots are not equal. The RGC data is indicated in orange and the OT data in blue. B: Pairwise correlation between the cone weight value for each trace, where each point in a plot represents a single trace. The magnitude of the weight is indicated in the x and y axes for the cone type indicated in the axis label. The overall correlation value is shown in the inset of each plot. RGC data is indicated in orange and OT data in blue. C: Average values for the histograms shown in A for the RGC and OT cone weights. The x axis represents the cone being analyzed and the y axis is the value of the weights. Each point shows the mean±SEM D: Cluster averages for the OT data set, showing the diversity of responses. Each row is a different cluster average and the x axis contains time, with all stimuli concatenated as shown in the symbols below. Color intensity represents the row-normalized ΔF. E: Cluster composition after clustering both the RGC and OT data sets together. The x axis contains each one of the 150 clusters found via a minimum in the BIC criterion, and the y axis contains the trace count (see Methods for details). Each bar is labeled based on how many traces from each data set can be found in that cluster. RGC data is in orange and OT in blue. F: Histogram of the purity indexes calculated for each cluster, namely what is the proportion of each data set in each cluster. Values close to 1 indicate mostly RGC traces are present in that cluster and values close to -1 indicate mostly OT traces.
RGC population responses discriminate intensity better than OT ones

Given the differences in individual responses between the two areas of interest, we then chose to assess these differences at a population level. One approach consisted in using unsupervised learning algorithms to calculate the degree of separation of the two data sets in an unbiased manner. Hence we used sparse Principal Component Analysis \(^\text{[169]}\) to reduce the dimensionality of each trace to its most relevant elements and then used Gaussian Mixture Models (via the Expectation Maximization algorithm) to group the traces in multi-dimensional clusters \(^\text{[18]}\). The OT data set cluster averages are shown in Figure 3.2.2 D as an example. Given the nature of clustering algorithms, responses are grouped by their similarity in high dimensional space, and hence if the responses for RGCs and OT cells are similar enough they will be grouped together. The composition of each cluster in terms of data set of origin can be seen in Figure 3.2.2 E. We calculated a cluster purity index where numbers close to zero indicate a mixed cluster and numbers close to 1 or -1 indicate clusters mostly assembled with traces from only RGC or OT data. The histogram of these indices for the data set can be seen in Figure 3.2.2 F. Here we show that most clusters are fairly pure, so the traces from each data set are well separated in high dimensional space. This indicates that OT cells are probably not acting as a relay for chromatic information, but there is probably a transformation of the chromatic signals in place, although the nature of this transformation is unknown.

As an alternative measure for this separation, we assigned clusters for both data sets independently and then built cross correlation matrices across stimuli as shown in Figure 3.2.3 A. The left panel shows the correlation between cone excitations in the stimulus, and the remaining panels show the correlation for each region. It is apparent that the stimulus correlation is fairly high at the two low wavelength LEDs given their spectral similarity (see 3.2.1 B). When looking at the two regions analyzed, it is clear that there is a higher degree of response decorrelation in the Tectum, especially the two lower wavelength stimuli, indicating an emphasis in this region. This decorrela-
Figure 3.2.3 (following page): Input decorrelation and loss of intensity information point to color processing between RGCs and Tectum A: (Left) Correlation matrix for the stimulus, comparing the excitation elicited on all four cones by each one of the stimuli (indicated in the axes). (Center and Right) Cluster average correlation across the entire data set for each one of the four stimuli as indicated in the axes. For all three panels, correlation coefficient is indicated by color intensity as shown in the color bar. B: Classification confusion matrices for RGCs, Tectum and the delayed RGC data set. (Top) Confusion matrices showing the performance of the classifier after being trained to predict the stimulus eliciting each response, using the cluster averages for training under 10-fold cross validation. Performance of the classifier is shown in the inset. The y axis shows the stimulus being shown and the x axis the classifier’s prediction. (Bottom) Using the same training procedure as before, the classifier was trained to predict the stimulus being shown and also its intensity. The resulting confusion matrices are shown with their respective performances. C: Similarity between the response profiles of each pair of animals by clustering all animals together and then separating the clusters per animal before calculating average pairwise correlations. D: Calcium kernels approximated from the RGC, OT and delayed RGC data, showing the matching of the latter two. The x axis shows time in seconds and the y axis is normalized intensity.
tion is observed to a lesser amount for the higher wavelength stimuli, but nonetheless it is higher than at the RGC stage. As mentioned above, a landmark of neural processing is the decorrelation of inputs from one stage to the next, so a high degree of decorrelation between RGCs and OT cells is expected if color information is being processed in any way. Additionally, this is congruent with the findings from the cone weight data.

Both of the aforementioned metrics offer a more unbiased but indirect measurement of information processing. To evaluate this processing more directly we used a supervised learning approach to ask which aspects of the color information can be best decoded at each stage. For this we used a multi-class SVM decoder trained on the neural responses within each region. The details of this decoder can be found in the Methods section.

If we label the data as each one of the four separate colored stimuli, both decoders perform almost perfectly, as expected given the high dimensionality of the data and the diversity of responses (shown in Figure 3.2.3 B Top). The differences arise when labeling the data as color and intensity values. As shown in Figure 3.2.3 B Bottom, the performance of the RGC classifier is better than the performance of the OT classifier (80% vs 40%). Since the calcium indicator is localized in the nucleus in the OT data and in the axonal terminals in the RGC data, we expected differences in their kinetics. To ensure this differences were not the main cause of the discrepancy in classifier performance, we also trained a classifier in an artificially delayed version of the RGC data, so that its approximated calcium response kernel matches the one from the OT data (see Figure 3.2.3 D). The delayed classifier performs worse than the non-delayed classifier (60% accuracy), but it still performs considerable better than the OT classifier, so the difference cannot be attributed only to differences in the kinetics of the response. In addition, to determine the chance level of classification, we trained the same classifiers under data with shuffled labels (Figure 4.3.7). The accuracies were between 1% and 4% for color & intensity, and between 20% and 28% for color only, confirming our results are considerable above chance.
Response profiles to colors are similar across animals at both anatomical levels surveyed

So far the discussion has been centered on the population data, but we have not addressed the contribution of individual to individual variability in the analysis, since highly divergent response profiles will change the conclusions that can be derived from the data. To obtain a compact, yet still comprehensive description of the response profile of each individual, we clustered all the OT data together, and then separated the traces and their assigned cluster per animal to calculate local cluster averages. These per-animal response profiles were correlated in a per cluster fashion and the average correlation between each pair is reported in Figure 3.2.3 C. As an alternative, we also clustered each animal individually and used an iterative algorithm to pair the clusters via correlation. This parallel method resulted also in predominantly moderate correlation between all pairs, as seen in Figure 4.3.5.

RGC and OT populations show selectivity for stimulus color and spatio-temporal structure

The differences established above regard only color information, but visual systems extract a large variety of information from the environment, including temporal and spatial patterns. To put our results in the context of this parallel extraction process, we presented the animal with a checkerboard pattern oscillating in total intensity in a sinusoidal fashion, moving vertical bars, and short flashes of darkness preceded and followed by an extended period of full field illumination. These stimuli were shown either in Red or UV to stimulate the four cones of the animal in as orthogonal a way as possible, all while reducing the stimulus set size due to experiment length limitations.

Figures 3.2.4 A and B show the cluster averages across individuals for RGCs and OT cells respectively. Two reciprocal approaches were used to quantify the similarities and differences of these profiles in terms of their stimulus selectivity. Either the clusters were grouped by which color elicited the maximal response for each one of the spatiotemporal patterns shown (Figure 3.2.4 C), or conversely, by which spatiotemporal pattern elicited the maximal response for each color (Fig-
In terms of the color of maximal response, the landscape seems to homogenize going from RGCs to OT cells, with a UV dominated RGC response but a more balanced OT response. This is observed across all spatiotemporal patterns.

In turn, there is a reduction in the response to Red flashes when going from RGCs to OT, while the UV flashes seem to elicit a stronger response in the latter. The checker responses seem to change as a complement to this, and hence the grating responses seem to remain stable. This indicates changes in the tuning of the cells from one stage to the next.

Stimulus color and spatiotemporal pattern decorrelate from RGCs to Tectum

As before, the response profiles shown allow for quantification of the differences in the response profiles to the battery of stimuli, but these differences are highly biased since the average was se-

Figure 3.2.4 (following page): Spatio-temporal and color channels decorrelate between RGCs and Tectum

A and B: Cluster averages of the responses from RGCs and OT cells under stimulation with Red and UV spatiotemporal patterns. These were a full field oscillating checkerboard, moving gratings and fast flashes of darkness. Each row is a different cluster average and time runs in the x axis. The stimulus at each time point is indicated by the symbols below the diagram. The line traces indicated the temporal structure of the stimulus. Color indicates the row-normalized $\frac{\Delta F}{F_0}$ as shown by the color bar.

C: Color that elicited the maximal response for each one of the spatiotemporal patterns for the RGC and OT data sets. Each row is an individual cluster and the patterns are indicated at the bottom. Color shows the preferred colored stimulus as shown on the side.

D: Spatiotemporal pattern that elicited the maximal response for each of the colors in each cluster. Each row is a cluster average and the colors are indicated in the x axis. The spatiotemporal pattern preferred is indicated on the side. For both C and D the cluster averages were sorted according to pattern for display.

E: Correlation matrices between the responses for each one of the stimuli and data sets used ((Left) is RGCs, (Center) is Tectum and (Right) is delayed RGC data). Each cell indicates the pairwise correlation between the concatenated cluster average responses for that given pair of stimuli. Color represents correlation as shown on the color bar. Note that the color scale is the same across the three matrices. CK: checkerboard, GR: moving grating, FL: flash.
lected as an arbitrary measure of characterization. As shown above, at a population level, it is ex-
pected to observe decorrelation of inputs as one progresses through processing pathways \[178\].
Hence, the response profiles were used to build cross correlation matrices for both data sets, includ-
ing also the delayed data mentioned in the previous section (Figure 3.2.3 D). Each matrix correlates
the responses to each one of the stimulus combinations. While checker and grating responses seem
to be correlated both in modality and color in RGCs, the flashes appear less so, with the UV flashes
anticorrelated to most of the other stimuli. Instead, in RGCs the correlation of the Red checker
goes down with respect to the other stimuli, while the anticorrelation shown by the UV flashes
turns into correlation. The overall magnitude of the correlation values across the matrix also goes
down. Finally, as before, the delayed data was unable to account for the decorrelation observed in
the OT data, in fact increasing the correlation between the checkered and grating stimuli. These
results point to a more complex interaction between the spatiotemporal structure of the stimulus
and color, highlighting the importance of the former in determining the usage of chromatic infor-
mation.

3.3 Discussion

In this study we have shown evidence that color information provided to the larval zebrafish is
present at the RGC and OT levels. We observed that all four cones seem to contribute in different
proportions, following previously described retinal spectral sensitivities. Additionally, we show
through several metrics that there is input decorrelation and loss of sensory information when go-
ing from RGC to OT. Finally, we observed differential processing of chromatic information across
spatiotemporal patterns, but varying levels of decorrelation depending on the pattern itself. Taken
together, our evidence suggest that given the changes this information experiences from one stage
to the other, there is potential for color processing in the larval zebrafish brain.
What additional features can be expected from the larval color vision system?

The evidence presented in this work is a first step into exploring the color vision system of the larval zebrafish. Given the evidence of differential processing of color signals, including their spatiotemporal pattern, the next step should be the search for color-selective receptive fields [39]. A hallmark of the color vision systems in place in primates and other vertebrate models [58], color opponent and double opponent cells are thought to be building blocks used by these nervous system to be able to compute color. Single color opponent cells show opponent responses to one cone type stimulation in their center and another in the surround. These cells are not in principle capable of discrimination of color [39], but they are thought to be a step in the process. Double opponent cells on the other hand will show selectivity for a particular subtraction of cone responses, both in their center and their surround (with the opposite valence in the latter) [222]. These cells have been incorporated in several models of color vision since they potentially allow for properties such as color contrast (the extraction of contrast from an image with homogeneous luminance structure), and color constancy - (the detection of a color as such, regardless of the background) [39, 41]. Previous work describes the presence of retinal units in the adult goldfish that fit this description [49]. Additionally, color constancy has been shown in the goldfish [223], and it is thought to depend on these cells [116]. This certainly opens the possibility for their existence in the larval zebrafish, and future research focused on color receptive field mapping, via an array of available techniques [49, 224, 225], should be able to confirm or deny this hypothesis.

Mapping of the 4-dimensional tetrachromatic space

The results shown above regarding the weights of each cone type into each RGC or OT unit come from an assumption of linearity in the way these responses are combined. There is evidence both of linearity, at least at the RGC level [226], and of non-linear features of the color landscape [227]. This question cannot properly be addressed with the stimuli used during this study, since gathering enough data to tile the putative 4-dimensional space would comprise a prohibitively large amount of time. Hence our conclusions are limited to the parts of this space that were covered, although based on the studies mentioned above, there has not been a description of large nonlinearities in
these landscapes and hence we expect it to be a smooth hyper-surface [227]. Future attempts at more thorough tiling might be made more efficient based on the color directions found to be more relevant to the animal [58], and also in more complete studies about the anatomy and physiology of the system that can guide the stimulus space selection. An alternative is that the space is less dimensionally rich, which is also a possibility that should be explored, but this will most likely require a behavioral component [74].

The need for color vision in the larval zebrafish

One aspect that should be discussed in the context of this research is the relevance of the developmental stage of the animal in the interpretation of the results [228]. Namely, given the larval zebrafish is still in a very active stage of development, there is a possibility that it does not have a mature enough visual system to perform as the adult [72], explaining the erratic results shown in the behavioral studies discussed [150, 151]. Given our results, not only the cellular components but the early stages of the color processing pipeline are active and functioning, and we know from previous research that the animal does react specifically to low wavelength light in a cone-dependent manner [229]. Hence we think that as a minimum a rudimentary wavelength discrimination system is in place [74]. Whether this is true for all wavelengths remains to be shown behaviorally, but a big caveat is that the natural relevance of color for zebrafish is still unknown, and hence it is difficult to guide research in this direction [90].

3.4 Methods

Fish rearing

5-7 days post fertilization (dpf) male and female zebrafish larvae (Danio rerio, Hamilton, 1822) were bred in a 14/10 hour light/dark cycle at 28°C in 10 cm dishes. The strains used were Tg(ath5 : SynaptophysinGCaMP6s, isl3 : SynaptophysinGCaMP6s) and Tg(HuC : H2BGCaMP6s), both in a nac/- background. The former expresses GCaMP6s at the axonal terminals of most RGCs in the
larval zebrafish, and the latter expresses the same calcium indicator in the nuclei of most neurons in the whole brain. All animal protocols were in accordance with NIH guidelines and the Harvard University IACUC.

**Stimulus presentation**

The stimuli were presented using a custom-built four channel projector. Briefly, two Lightcrafter projectors (Lightcrafter Evaluation Module, Texas Instruments, Dallas, TX, USA) were aligned to project co-axially. One of them was modified to support projection of LEDs centered at 606, 463 and 397 nm by changing the dichroic mirrors in the light engine. The second projector was stripped of its dichroic mirrors and the focusing lens was replaced by one supporting UV transmission (354-330-A, f=3.1 mm, NA = 0.68, Unmounted Geltech Aspheric Lens, Thorlabs Inc., Newton, NJ, USA). Then a UV LED (365nm, Mouser Electronics, Mansfield, TX, USA) was mounted at the entrance port of the former red LED. The two projectors were coupled using a flat mirror and a dichroic mirror (PF20-03-F01, Thorlabs Inc.) and then their projections were aligned underneath the animal. All the stimuli were synthesized by custom software written in LabVIEW (National Instruments, Woburn, MA, USA).

**Calculation of cone contributions**

This first approximation was calculated assuming linearity in the signal summation from different cone types. Namely, that a linear combination of the contributions from each cone type, weighted by constant factors, is able to explain the results of the study. To perform this analysis, the Fourier transform of the traces was extracted to get the power at the frequency of stimulation (0.125 Hz). This power was used as the value for the response for each LED. Then, an interaction matrix was constructed, containing the expected excitation of each cone type based on the cone spectra, the LED spectra and the power of the LED. Hence, for each stimulus, there is a matrix characterizing the response. The key element is that, for each stimulus, each LED is turned on on its own, and hence the entire relationship reduces to a four equation, four unknown system that can be solved
numerically. This will yield a set of cone "weights" for each trace.

\[ R_1 = C_1 * L_1 + C_2 * L_1 + C_3 * L_1 + C_4 * L_1 \]
\[ R_2 = C_1 * L_2 + C_2 * L_2 + C_3 * L_2 + C_4 * L_1 \]
\[ R_3 = C_1 * L_3 + C_2 * L_3 + C_3 * L_3 + C_4 * L_1 \]
\[ R_4 = C_1 * L_4 + C_2 * L_4 + C_3 * L_4 + C_4 * L_1 \]  

(3.1)

Here, the cone weights are represented by C and the subscript represents the cone type. R are the calcium responses for each one of the stimuli, indicated by the subscript. Since each LED is turned on only for one stimulus, then the excitations elicited in each cone type are represented by L, with the subscript indicating which LED is one.

**Brain imaging**

All the 2 photon calcium imaging was performed in a custom built point scanning 2 photon microscope. Briefly, a Mai-Tai tunable femtosecond laser tuned to 950 nm was passed through a computer controlled half-wave plate and a polarization sensitive. The side port of the prism was aimed at a beam dump, so that the amount of energy into the system could be regulated based on the relationship between the angle of beam polarization, and the selection angle of the prism (extra power would go to the beam dump). After the prism, the beam was expanded to 5 mm and delivered to the center of a set of scanning galvanometric mirrors (Cambridge Technology, Cambridge, MA). The beam is then scanned by the mirrors through a scan lens and a tube lens so that expansion covers the back aperture of the objective. The pivot point at the mirrors was imaged at the back aperture of the objective so that motion of the beam at this point is minimal. The objective used was an Olympus 20X XLUMPLFLN-W water immersion objective (Olympus Corporation, Shinjuku, Tokyo, Japan).

Light collected from the sample was relayed to a dichroic mirror that then diverted it to a gateable photomultiplier tube (H11526, Hamamatsu Photonics K.K.), after bandpassing by a filter (Chroma, Bellows Falls, VT). Additionally in the path, there was a Hitachi camera focused on the
sample plane to allow for rough sample positioning. As with the stimulus presentation, the entirety
of the microscope control software was written in LabVIEW (National Instruments, Woburn, MA, USA).

Images were acquired at 2 frames per second, at a resolution of 320x320 pixels. Each trial was
40 seconds in length with 10 seconds of adaptation, 20 seconds of stimulus presentation and 10
seconds of rest before the next trial. All stimuli were shown in triplicate to each animal before
moving onto the next z section and repeating the whole process.

**Data pre-processing**

The raw data followed a pre-processing pipeline has been described previously. Briefly, the raw
imaging data was imported into Matlab (Mathworks, Natick, MA, USA), and all frames within a
z section where aligned based on their cross correlation from time point to time point. Following
alignment, the $\Delta F/F$ was calculated for each stimulus repetition and the repetitions were collapsed
into an average. With the repetitions condensed, all the frames corresponding to one z plane were
concatenated and the correlation of each voxel with its eight neighbors within a plane was calcu-
lated across time. An iterative algorithm was used to find the highest correlation value in each z
plane and then start grouping it with its neighbors based on a correlation threshold, up to a size
threshold or until there are no more neighbors fulfilling the correlation threshold. These small
groups of voxels, termed units, were then used as the unit to generate each one of the traces used
in the study. To obtain each trace, the traces from each voxel in a unit were averaged together. Fi-
nally, signal-to-noise ratio was approximated as described in Baden et al. [18] by taking the ratio
of the averaged standard deviation across repetitions for each trace and the standard deviation of
the average. Then traces below the 25th percentile where excluded from the analysis (around 10% of
the traces).
**Delayed dataset calculation**

To artificially delay the time constant of the calcium indicator in the axonal data, the calcium kernel of each data set was approximated by the calcium responses when switching from one stimulus to another. Since these transitions were discrete, they elicited a clear spike of activity in most light-responsive cells. Averaging this response across traces and stimulus presentations yielded a calcium kernel similar to the one reported for nuclear GCaMP6f expression ($\tau \sim 0.4$ vs $\tau \sim 0.33$ respectively Kawashima et al. [160]), as would be expected from the slower GCaMP6s [157]. Once the calcium kernels were calculated, an exponential kernel was calculated so that convolution of the axonal data with it yielded a similar decay than the one observed in the nuclear data ($\tau \sim 0.4$). This is the data used as the delayed set.

**Dimensionality reduction and clustering**

Given the high dimensionality of the data, the traces were processed using the method described in Baden et al. [18] for their own traces. Sparse principal component analysis (via the SpaSM Matlab package by Sjöstrand et al. [230]) was used to reduce the dimensionality of each trace. In particular, eight sPCs were used per stimulus, each one with ten active bins. To find the ideal number of clusters the analysis was performed with several cluster numbers and then the one with the minimum at the Bayesian Information Criterion was selected. This criterion balances the increased in fit with a stronger model with a penalty to the complexity of the model. The entire process was performed using the Expectation Maximization algorithm in Matlab to fit a Gaussian Mixture Model to the data.

**Clustering of the combined data set**

To assess the overall similarity between the responses obtained from the RGC and OT data sets, all traces were concatenated into a single matrix and then clustered using the method described above. Then, a purity (or preference) index was calculated to assess how much of each cluster belongs to
either of the data sets.

\[
Purity \ Index = \frac{\#RGC \ traces - \#OT \ traces}{\#RGC \ traces + \#OT \ traces}
\]  \hspace{1cm} (3.2)

A histogram of these indexes is shown in the text.

**Data set correlation**

For calculation of the data set correlation matrices, the data sets were reshaped so that all of the responses for a given stimulus are concatenated, and separated by stimulus. Then a correlation matrix is calculated based on this input so that the result has the dimensions of the number of stimuli. This was done with both the four LED data sets and the two LED + pattern data set. For the stimulus correlation matrix, the calculated excitations for each one of the cones under each stimulus was treated in the same way as the different traces in the calculation above. For calculation of the fish to fish correlations instead, there were two separate approaches. In the first one, the data from all the animals in the OT data set was clustered together, then the traces from each animal were averaged based on their belonging to a cluster. These averages were then correlated across animals and an average correlation per individual was calculated. The second approach involved clustering each animal separately, calculating cluster averages, and then pairing these averages across animals based on correlation. The pairing was performed by calculating a pairwise matrix correlation for each animal’s set of cluster averages, and then pairing clusters from each by starting at the highest correlation in the matrix. The two paired clusters were eliminated (the row and matrix of the pairwise matrix) and the process was repeated. Then an average correlation between two animals was calculated as the average of these pairwise values.

**SVM color and intensity classification**

To perform the Support Vector Machine classification, the data was supplied to the fitcecoc function in Matlab, which trains a series of binary SVM classifiers to perform multi-class classification. The structure of the binary classifiers is a one-vs-all arrangement, were each binary classifier is
trained to separate one category from all the rest lumped together. Then the points that score the highest for a given category are assigned to that one. One classifier was trained on the four LED sinusoidal data to discern the four LEDs (i.e. four categories). Only the stimulus period was included, and the data sets trained were the RGC, OT, and delayed RGC ones. The second classifier was trained on the same data sets, but instead of separating the data per stimuli, it was separated by stimuli and also four intensity levels. The classifier was ran with 10-fold cross validation to prevent overfitting. Performance of the classifier was assessed as the average percentage of the traces across the diagonal of the confusion matrix (diagram comparing the delivered vs predicted stimulus).

**Stimulus selectivity indexes**

After clustering, each trace was classified depending on which color or which spatio-temporal pattern elicited the maximum response. This yields a reduced, 2- or 3-number vector for each trace. These vectors were sorted and plotted as an image to show the distribution of both the RGC and OT data sets.
4.1 Future directions

Sensorimotor transformation in UV avoidance

As presented in Chapter 2, the larval zebrafish show a form of wavelength dependent behavior, where they weakly avoid UV light, and this avoidance seems to act more strongly when UV light is mixed with visible light wavelengths. Although the behavioral description of this behavior is rather informative, currently there are only neuron-level descriptions of phototaxis, a related non-chromatic behavior. These are both the work by Burgess et al. \[136\] and Zhang et al. \[138\] mentioned above. In the former, the ON retinal pathway is thought to trigger forward swims towards
light and the OFF pathway to drive turns away from darkness. More recently, the latter describes an involvement of the dorsal left habenula and AF$_4$, but the full sensorimotor transformation, and any involvement of color, are still unresolved.

One of the motivations for the research in Chapter 3 of this work, was to lay the foundation for tackling this question. There were a number of findings relevant for this endeavour: It was confirmed that the retinal output of the animal is indeed dominated by short wavelength signals at this age, as was described in ERG studies in the past [111]. It was also confirmed, as could have been inferred from bipolar cell connectivity [113], that the signals coming out of the retina are not pure color channels, but are generally complex mixtures of the different cone types. More importantly, it was shown that these mixtures were indeed able to code differentially for the color stimuli used, which indicates the information for color-based decisions is at least present at the retinotectal interface.

Taking the above into account, a plausible mechanism for the UV avoidance behavior would be the comparison of UV-containing and non UV-containing channels at a later stage of processing, so that the amount of UV light can modulate the response to visible light (as has been described for opponency mechanisms [206]). Normally this response is of positive valence [136], but if enough UV light is present then the valence would be reduced and the turning behavior would be more neutral towards the light source. As shown in Chapter 2, UV light alone elicits only weak avoidance behavior, meaning that this valence modulation might be divisive (as opposed to subtractive), so that medium-to-high overall light levels are required for it to have a discernible effect in behavior. Conversely, it could also mean that the signal cannot take strong negative values.

The site of this putative computation is currently unknown, although likely candidates are the OT or the targets of the AFs [25, 30, 138]. As shown in Chapter 3, the signals required for this computation are available at both of these stages, so a concomitant imaging and behavior experiment will be required to pick out the relevant neural responses. This has proven difficult given the immobilization of the animal required for imaging. This reduces the overall bout frequency of the fish [134], and in a behavior like phototaxis with low baseline bout frequency and also fast adaptation (see Chapter 2) this results in extremely few movement events. There have been circumstantial reports of the paradigm working more effectively in a fictive preparation [216], but there is a lack
of published evidence in either direction and bout frequency is also reduced in the latter.

COLOR ASSAYS AND INNATE BEHAVIORS

A more general problem that applies to color vision research in the larva, is the lack of described color-dependent behaviors of any kind.

In particular, there is a clear lack of an innate behavioral paradigm that depends on wavelength. As described in Chapter 1, classic color vision science has traditionally aimed for the use of training paradigms for assessing color vision. This allows the experimenter excellent control over the variables in the stimuli, and has proven successful across many organisms, including primates, reptiles, fish, crustaceans, and insects. The training paradigm history with the larval zebrafish has been unsatisfactory at best, since the few assays that have been published have not been followed up or replicated. Additionally, there is evidence that standard training paradigms in the animal will not be successful given its developmental stage, as shown by Valente et al. In this publication the authors attempt classical and operant learning paradigms at different ages in the larva, and show that there is a proportion of the larvae that learn earlier, but this seems to be a random subset of the overall population. The population does not become homogeneous until around two weeks of age, well past the age where the animals can be imaged at a whole brain level. While this does not preclude the possibility of a working learning paradigm on the larva, it certainly discourages future searches in the subject matter, and several groups are already attempting imaging in older animals to circumvent this obstacle.

With learning paradigms out of the question, the remaining option is the search for an innate behavior that depends on wavelength. As mentioned in earlier chapters, there have been attempts at assessing the innate color preference of the larvae, either via colored foods or place preference assays, but the results from these studies are inconclusive and the stimuli are ill defined, especially since in the case of place preference the animal is presented with large, static fields of a given color, which should inhibit color perception if the larval system is anything like the human system.
A tempting alternative would be the use of moving gratings, given the robustness of the OMR behavior in the larva. This option was discarded by Orger and Baier [152] when they measured the color contrast abilities of the larva. By using the OMR, they showed that the larvae are red-green motion colorblind, meaning they are unable to generate color contrast between red and green when they are equiluminant in a moving grating. This is the same in humans and other animals [234, 235], and derives from the parallel channel structure of the retina, where the motion signals are computed disregarding their color inputs, and hence the channel is effectively color blind [47].

Although this seems like a bleak landscape, one of the reasons the larval zebrafish has risen as a popular model for neuroscience is its wide behavioral repertoire [100, 236]. While phototaxis and OMR might not be ideal paradigms for the search of color discrimination, there is a slew of behaviors that to date have not been tested for color vision contributions. Plausible candidates include prey capture [119, 141, 143], looming responses [145] and flashes of light [139]. All of these are highly visual behaviors that have already been partially characterized in terms of circuitry and that could potentially involve color vision components.

Hence, an attractive future goal would be to capitalize on the existing descriptions of these behaviors, and screen them for color vision components, given the visual stimulation system already exists. In particular, prey can be simulated by the presentation of small moving dots, as described previously [141], and looming and flash responses can be also modulated in color while keeping the other parameters of the stimulus constant. The finding of a color dependent behavior that also is effective in an embedded preparation will open new avenues in the study of color-related sensorimotor transformations.

Understanding the evolutionary drive behind tetrachromacy

An intriguing fact about the zebrafish visual system is the presence of four spectral types of cones. Modelling efforts support the optimality of trichromacy for extraction of information from natural scenes [237]. The addition of cones is not deleterious, but because of the necessary increase in the overlap in their spectra as more cones are added, it becomes less and less beneficial to have extra cones. An exception to this can be observed in some reptiles and birds. These species express
screening pigments in the form of colored oils in front of their cones [77]. These pigments act as high pass filters that narrow the spectrum of the cones they shadow, and hence the optimality of a tetrachromatic retina increases. This is not the case in zebrafish, making the evolutionary survival of its four cone types a mystery [77].

The null hypothesis to this problem comes from the genetic history of fish. Teleosts have experienced at least two full genome duplication events in their evolution, which has been theorized as the generating factor for so many pigments [238]. In fact, recently the zebrafish genome was scanned for photopigments and it was shown that it has around 42 of them expressed throughout the body [12], making almost every tissue in the adult photosensitive. Considering this overabundance of pigments, can the explanation for having four visual pigments be that a number of them might be under neutral selection, and do not accomplish any functional role anymore?

A counterargument to this hypothesis is a study by Sabbah et al. [208] where the complexity of the spectral composition of natural scenes was evaluated by PCA, both in terms of irradiance of the light source illuminating them and also in terms of the reflectance of their different components. By matching the effectiveness with which the human visual system analyses terrestrial images to the one from the fish for underwater images, the authors were able to show that indeed, the shallow water animals need more PCs to be able to explain the scenes in the same way humans do terrestrial images, justifying the presence of the additional cone types.

This model offers a very enticing possibility for the zebrafish, since its natural habitat is shallow small rivers or rice paddies [90, 91], with exactly the complexity in the environment described above. Hence, considering the knowledge acquired in this work and the potential of the system, it would be of great biological interest to approach this question. Namely, brain signals can be measured upon presentation of natural images to the animal, made to match the spectral composition of the zebrafish’s habitat as measured independently via hyperspectral imaging [239]. The brain patterns obtained can be analyzed in the same way shown in Chapter 3 to see how optimal is the information extraction/decorrelation, and whether removing a channel, for example with a cone-type mutant, impairs this process.
4.2 **Discussion**

**What does color vision look like in the larval zebrafish?**

Color vision has been the subject of this work, and of several research publications in the past, some of which are mentioned in Chapter 1. If there is something this accumulated evidence has told us, is that despite the widespread presence of color vision in the animal kingdom \([59, 240]\), the nature of these systems seems to be very specific to each group of organisms. In particular, it does not seem plausible that, given the diversity in brain structures and connectivity across species, all color vision circuits operate in the same way \([74]\). Namely, it is thought that the locus of hue is somewhere in area IT/V4 in the human brain and also in the monkey \([241–243]\), but there are several other organisms with functionally equivalent color vision that lack these structures \([65, 244]\), or a cortex altogether as is the case with fish \([73]\).

Kelber and Osorio \([217]\) propose a systematic approach to this matter: four well defined levels of color vision, which should be able to categorize the known systems. Briefly, level 1 is comprised of sensitivity to wavelength in the illumination, such that a behavior like UV avoidance can be implemented \((196, \text{Chapter 2})\). Level 2 involves what are termed wavelength specific behaviors, where the color signal is also integrated with form, so that the behavior is driven by interactions with objects or conspecifics at particular wavelengths. This is observed in some species of butterfly and their selection for egg laying sites for example \([245]\). Note that his integration with form requires spectral comparisons in a scene, and hence at least a dichromatic system. Level 3 is what has been observed in most animals considered to have conventional color vision, which is the possibility of learned behaviors dependent on chromatic information. This implies the existence of a neural representation of color, and examples abound \([64, 232, 246, 247]\). Finally, level 4 comprises not only the capability for learned behaviors, but also of categorization and hence generalization of chromatic information at a neural level. This has been observed in humans \([248]\) and birds \([249, 250]\), but it hasn’t been shown conclusively in primates \([251]\) or other organisms \([217]\).

Intuition temptingly leads to using the number of visual color pigments as a proxy for color vision system complexity, but as comparative color vision has shown, this is extremely misleading.
An umbrella reason would be the early evolutionary appearance of color visual pigments, so that even very early organisms had several visual pigments [54]. There are also many species-specific examples of why this notion is misleading. One such case is the mantis shrimp. This stomatopod rose to fame because it expresses up to 12 opsins in its eyes, and was hence thought to have excellent color vision, unparalleled in vertebrates or invertebrates. Work by Thoen et al. [68] unveiled that in fact the color discrimination abilities of this animal are fairly poor, and nowhere near the levels it should achieve based on its rich opsin expression. Then it was proposed, and partially confirmed via a model, that the mantis shrimp has a completely different system for color vision, where it uses scanning head movements to get the same scene in an array of the different photoreceptors [69]. This results in a pattern of activation that determines the identity of the color, much like a winner-takes-all mechanism. At the other extreme are animals, like some species of jumping spiders, that despite having a single photopigment are able to generate color vision by stacking their receptors in the axis of the light rays, and relying on chromatic aberration to separate the wavelengths of light [252].

Zebrafish larva clearly fall at least within Level 1 of the Kelber and Osorio classification, but then, their position in Level 2 or above remains to be shown. This seems like a difficult task, especially given the mentioned apparent absence of learned behaviors in the larva. As mentioned above, the neural signal for Level 3 would be a representation of hue somewhere in the brain, so not only are cone signals used to detect a particular wavelength, but in fact they are combined to compute hue [218]. The answer to this problem will lie then in a much deeper interrogation of the system, and even then it might be hard to define whether the existence of this computation alone has any meaning, given it could lack a behavioral function until much later in the life of the animal. The point discussed here is born from the unparalleled access the larval zebrafish grants to a full color vision system [105, 166], and hence future findings will most likely reshape the definitions of color vision systems in the natural world beyond behavior and neural circuits in isolation.
Lack of plasticity in the larval zebrafish

The results shown in Chapter 3 point to a very homogeneous response profile to color across fish. This is true to the point where, for both of the stimulus paradigms used, every cluster was present in almost every animal. This is also true for non-color stimuli (Clemens Riegler, personal communication) under a much broader battery of spatiotemporally patterned stimuli. The latter is also supported by earlier anatomical findings, where the connectivity of most visual structures is generally extremely hard wired to give a defined overall pattern, both at the level of RGCs and OT [25, 98, 122, 253]. This neural hard-wiring is also supported by the apparent scarcity of learned behaviors in the larval animal. As mentioned in the previous section, there are currently no consistent learning paradigms in the larval zebrafish, other than gain adaptation in a feedback paradigm [254, 255] and habituation to repeated sensory stimulation [256], while adults can be readily trained [72, 257, 258]. All of this evidence points to an organism that is clearly in development, and seems to show very basic functionality.

This is in stark contrast with the complexity of the innate behaviors in the zebrafish. As was reviewed briefly in Chapter 1, there is a large array of behaviors that the larva is able to perform almost as soon as it can swim [133, 136, 139, 141, 145]. These involve highly coordinated actions over extended periods of time, many of them involving quick processing of sensory information and then execution, way beyond what would be considered a reflex. A prime example is prey capture behavior [119, 141, 143], where hunts are composed of very complex sequences of tail and fin movements. These align the fish in real time to a moving prey, and then strike said prey. Even simpler behaviors like the OMR also involve fairly complex processing of visual information and selection of motion actions [121, 133].

Hence, it is somewhat surprising that, given the potential for very fast development, learned behaviors have a delayed onset in the larva (around two weeks [228]). This could be due to the ecology of the larval zebrafish in its natural environment. Namely, as it has been described [91], zebrafish eat their own eggs [92], even if laid by the same adult moments before. Hence the larvae face a very hostile environment shortly after hatching, which would justify wiring the system for basal, yet complex, behaviors as fast as possible to guarantee survival (visually evoked escapes,
maintenance of position in streams, prey capture, etc). Additionally, given the size of the larva, its environment is fairly homogeneous in the first days of life, as opposed to the juvenile and adult life where there is a much more significant amount of foraging, covering larger distances [90, 91]. This argues for very little utility of learned behaviors in the very early stages of development, and is consistent with the findings so far, both in terms of the wiring of the system and its capabilities. Testing this hypothesis is beyond the scope of the current work, but the results shown and the existing literature prompt for their discussion in this section.

**Ultraviolet vision in the zebrafish**

Ultraviolet light has generally a negative connotation among humans, given its ability to cause mutations in DNA and its abundance in the spectrum of solar radiation, aside from the fact that it is invisible [259]. As mentioned in Chapter 2, it was early on that Lubbock [189] discovered that ants were able to detect this seemingly invisible radiation readily, and during the course of the 20th century many more animals were discovered to use ultraviolet light in one way or another [191, 192, 194, 260–262]. The role of UV vision in the larval zebrafish was discussed briefly in Chapter 2, but the findings discussed in Chapter 3 should be put into this context and discussed further.

As mentioned before, the UV visual pathway has a cryptic role in the life of the larva, given that zebrafish do not have strong pigmentation, and there is very little data about the amount of UV light present in their natural environment [90]. The discussion in Chapter 2 suggested that the UV avoidance behavior observed is relevant in keeping the larva in the appropriate light environment, but as it was pointed out there, this does not explain the image forming part of it, given that pure detection can be achieved with a non-visual system altogether. So, why have the UV pigment in the cones?

One alternative is that it is a vestigial trait, coming from the evolutionary history of visual pigments. As mentioned above, cone pigments originated in their current spectral varieties before rod pigments appeared [54]. Hence, ancestral organisms expressed most of these. Therefore, its presence in the zebrafish could be as a remnant of the original pigment set. The issue with this idea
is there is evidence that the pigment array of different organisms is a relatively fast evolving trait \[263\] and hence there has been enough time for the animal to have lost pigments that are not of selective relevance. This in fact indicates that the UV pigment probably plays an important role in the life of the zebrafish. Additionally, UV sensitive pigments are already expressed in most of the zebrafish body, so the argument for the UV pigment appearing first in the eye, and then staying there because of its relevance in UV avoidance does not apply either \[12\].

This evolutionary knowledge is congruent with the evidence presented in Chapter 3. Namely, among other findings, this research confirms that, as suggested by earlier ERG measurements \[111, 215\], UV light is very relevant in terms of neuronal signals. We observed a great amount of UV light sensitivity in the system, and it seems to be involved in most visual channels, be it motion, fast kinetics stimulation or just full field stimulation, both in ON and OFF modalities.

Going back to the behavioral study, the expectation for the circuitry supporting a specialized avoidance behavior would have been a dedicated pathway to drive the avoidance response, and a more reduced presence of the UV signals in the entire system. Given this is not the case as discussed above, then there must be an additional role of UV light in the visual input the zebrafish receives. Considering the widespread distribution of the UV signals, it would seem that UV light might be playing a passive role in the vision of the animal, expanding the range of light the animal is sensitive to. Other options mentioned in Chapter 2 remain possible, namely prey capture \[141\], polarization \[214\] and vertical navigation \[264\]. All of these, together or in conjunction, would be compatible with the results obtained in Chapter 3, with the only caveat that if any of them are to be specifically dependent on the UV signal, then downstream circuitry should be able to compute the amount of UV content in the inputs, as posed in the Future directions section above. No such site has been found yet, but most of the brain has not been surveyed in this study so their existence is perfectly likely.
4.3 Concluding remarks

The work presented here is the first step into the largely unknown world of zebrafish color vision, and in general into a vertebrate system combining full access to color-driven brain signals and complex behaviors. Hence, there is a large amount of work to be done, but the findings derived from this study allow, as somewhat depicted above, for the construction of road maps to tackle questions that were hard to approach or even hard to formulate in the past. Part of the reason for this is that color vision elicits a highly relevant, yet highly subjective influence in our perception, making the first step in color vision research the decoupling from these intuitions and the allegiance to the scientific method and open thinking, for nature will certainly continue to pose unexpected solutions to equally unexpected problems.
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Appendix

Supplementary Material for Chapter 2

This section contains the supplementary figures referenced in Chapter 2.

Figure 4.3.1 (following page): Cone excitations for each stimulus and bout parameters for Figure 2.2.1 A: Cone excitations from each of the stimuli used in the study. The solid columns on the left show the stimuli as they are shown in the respective figures (indicated at the top of each panel), including the power density measured at the sample (red, blue and magenta bars represent the 3 LEDs used in the study). The spotted columns on the right show the relative excitation to each one of the zebrafish cone types from these stimuli. The L (red), M (green), S (blue) and UV cones are represented by the red, green, blue and magenta bars. Black bars represent no stimulus. These excitations were calculated by convolving the emission spectra of each LED at each intensity by the modeled absorption curves of each cone as shown in Figure 2.2.1 B. B(i-iv): Bout parameters for the phototaxis experiments shown in Figure 2.2.1 Ei-iii. There was no apparent trend in any of them, either depending on stimulus or light intensity (gray represents the rest stimulus and has more bouts because there is a rest in between every trial). Values are mean±s.e.m..
Figure 4.3.2 (following page): Average bout parameters for the larvae during each stimulus in Figure 2.2.2 A(i-vi): Displacement per bout, Bout peak speed, InTer-Bout Interval, Latency to peak speed, Bout duration and Number of bouts averaged for each stimulus in Figure 2.2.2 A. Gray represents all LEDs engaged on both sides (which is the rest stimulus, shown in between every other stimulus and hence showing more bouts than the others), black is LEDs off on both sides, and the other stimuli all represent a color on one side and black on the other side. As shown, there is no discernible modulation of either of this parameters based on stimulus. B(i-vi): Same kinematic parameters outlined for panel A but for the experiments in Figure 2.2.2 B(i-iii). The stimuli showing the replacement of darkness with UV are shown in A and the stimuli that merge in UV in the color side are shown in B. There is not significant modulation of the response, either from stimulus or light intensity.
Figure 4.3.3 (following page): Average kinematics of the chokh and TeNT larvae during experiments A(i-vi): Displacement per bout, Bout peak speed, Interbout interval (ITBI), Latency to peak speed, Bout duration and Number of bouts averaged for each stimulus in Figure 2.2.3 A. The red symbols show the mutant fish and the black symbols the wild type siblings. Although the wild types show more bouts in average, the bout statistics for the fish seem very similar. B(i-vi): same parameters as in A for the TeNT larvae. Red symbols show the TeNT-expressing larvae and black symbols their wild type siblings. No discernible difference was observed between conditions or between stimuli.
Figure 4.3.4: Preference index shown by larvae during full field stimuli A(i-iii): preference indices calculated from the full field stimuli experiments. This shows the expected response of no preference given there is no difference between the sides.
Supplementary Material for Chapter 3

This section contains the supplementary figures referenced in Chapter 3.
Instead of clustering all the animals together, the data from each larva was clustered independently and then the clusters were paired up via correlation as described in the methods.
Figure 4.3.6: Shuffled cone weight correlations To control for possible unforeseen biases in the calculation of cone weights, we shuffled the data only across the rows and calculated the correlation coefficients between units for each cone type. This destroys the correlation between values in a unit, but keeps the population of values the same across all units for each cone type.
Figure 4.3.7: Classifiers trained on shuffled data To control for the performance of the classifiers used to evaluate encoding of the stimulus at each stage, we utilized the same training software but we randomly shuffled the labels of the data. The results are shown in the figure. A and B: confusion matrices for the 4 and 20 category classifiers using the OT data. Accuracy of the classifier is shown in the white rectangle. C and D: same as above for the RGC data.
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