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A Red Herring in the Zebrafish Retina:**Regional Specializations among Retinal Ganglion Cells in Zebrafish Larva linked to
Chromatic Encoding of Prey Stimuli**

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Thesis dissertation for doctoral degree in systems neuroscience

by John Bear

at the University of Sussex, Brighton, U.K.

This thesis was officially submitted on September 30, 2021

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DECLARATION

I, John Bear, hereby certify that this thesis, which is approximately 45,000 words in length, has been written by myself, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree. I declare that this thesis has not been and will be submitted in whole or in part to another university for the award of any other degree. However, the thesis incorporates to the extent indicated below, in the results presented in chapters 2-5, material already submitted for publication in the journal *Current Biology*. This work has been conducted partly in collaboration with researchers in the Baden laboratory, at the University of Sussex, and with those of the Semmelhack laboratory, at the Hong Kong University of Science & Technology, as indicated in the acknowledgements below.

I was admitted as a research student in July of 2017 and as a candidate for the degree of Doctor of Philosophy on December of 2017; the higher study for which this is a record was carried out in the University of Sussex between autumn 2017 and summer 2019, and in the Hong Kong University of Science & Technology between autumn 2019 and summer 2020.

I would also like to add that in spring 2020, on account of the shutdowns in Hong Kong due to the covid-19 pandemic, I was ultimately unable to continue my research work or even to remain at HKUST. Cutting short my research by approximately 12 months meant that some of the work which would have gone towards linking chapters 5 and 6 content remained unfinished. The experimental methods I would have pursued during the academic year 2020/2021 are detailed in the discussion section of chapter 6, in which I describe potential options for future research in functional and behavioral prey capture paradigms.

Date Signature of candidate

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ABSTRACT

In the study of vision and visual ecology, the larval zebrafish is a widely used model organism for speculative and translational research due to its genetic accessibility, amenability to non-invasive *in vivo* recording techniques, and repertoire of well-studied and stereotyped visually guided behaviors. Yet, although a great deal is known about zebrafish outer retinal circuitry and visual brain areas, the retinal ganglion cells (RGCs), which transmit information from photoreceptors to arborization fields in tectum and pretectum, remain incompletely understood. In particular, recent research has demonstrated the existence of a specialized area within the ventrotemporal region which appears to serve as a prey detection unit, but how the inner retina computes prey-related signals is unknown.

In this thesis, we use *in vivo* two-photon imaging and photolabeling to create an anatomical and functional profile of ventrotemporally-positioned RGCs. We also compare the structure and physiology of this neuronal population to those in other retinal regions, and use the results of our analyses to probe chromatic preferences of the 7 *dpf* larval retina in terms of kinematic output. We show that the function, distribution, and morphologies of ventrotemporal RGCs differs substantially from the rest of the retina, and that their feature-response set appears to match chromatic components of prey-like stimuli capable of eliciting hunting behaviors. Taken together, our results strongly suggest that prey-responsive RGCs viewing the upper-frontal visual field are highly specialized for short-wavelength chromatic computations necessary for detecting prey stimuli in natural settings.

LIST OF ABBREVIATIONS

AC – amacrine cell

AZ – acute zone

B – blue

BC – bipolar cell

BIC – Bayesian information criterion

D – dorsal

dpf – days post fertilization

G – green

GCL – ganglion cell layer

GMM – Gaussian mixture model

hpf – hours post fertilization

INL – inner nuclear layer

IPL – inner plexiform layer

N – nasal

ONL – outer nuclear layer

OPL – outer plexiform layer

PCA – principal component analysis

PR – photoreceptor

SD – synaptic density

R – red

RGC – retinal ganglion cell

T – temporal

UV – ultraviolet

V – ventral

1. GENERAL INTRODUCTION

1.1 Paley and Darwin: The evolution of an idea

In 1804, as part of his argument for a natural theology based on the appearance of design in nature, William Paley famously likened the eye to a product of intentional human contrivance, namely, a telescope. He drew attention to the fact that both telescope and eye are exquisitely fashioned and adjusted to accommodate the transmission and refraction of light in focusing an image on the eyepiece and retina, respectively, remarking that “there is precisely the same proof that the eye was made for vision as there is that the telescope was made for assisting it” (Paley, 1804, 16). The first example he gives in defense of this thesis is that of the fish lens, which is more nearly spherical than it is in terrestrial animals, an adaptation which allows photons passing into the eye from the relatively thicker water medium to be refracted by a sufficiently convex surface to project it onto the photoreceptor cells. Going on to cite several other modifications of the fish’s eye enabling it to accurately detect waterborne versus airborne light, Paley concludes by asking, “What plainer manifestation of design can there be than these differences?” (Paley, 1804, 16).

Half a century later, Charles Darwin was to plant the seeds of an alternative hypothesis for how such “manifestations of design” might arise, one with more appeal to the scientific community than Paley’s had since it did not invoke a supernatural agent working from outside the natural order. Darwin too noted that the eye was analogous to a telescope, but dismissed the inference of intelligent design as presumptuous, declaring instead that biological systems are the product of small, cumulative changes driven by selective pressures.

To suppose that the eye with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection, seems, I freely confess, absurd in the highest degree. When it was first said that the sun stood still and the world turned round, the common sense of mankind declared the doctrine false; but the old saying of *vox populi, vox Dei*, as every philosopher knows, cannot be trusted in science. Reason tells me, that if numerous gradations from a simple imperfect eye to one complex and perfect can be shown to exist, each grade being useful to its possessor, as is certainly the case; if further, the eye ever varies and the variations be inherited, as is likewise certainly the case; and if such variations should be useful to any animal under

changing conditions of life, then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable by our imagination, should not be considered as subversive of the theory (Darwin, 1859, 144).

Darwin's original ideas about natural selection, common descent, and speciation have matured into the field of evolutionary biology. One of the more recent players to emerge onto this field, visual ecology, has taken up the challenge posed by both the theologically minded Paley and the naturalist Darwin. With its formalized inception in the 1930s, visual ecology began to examine in detail how animal eyes are adapted to their ecological needs given the constraints imposed by body plan, physiology, and metabolic and environmental resources.

Visual ecology research seeks to address three questions. In the first place, it asks why gross anatomical differences arise in the eyes of different animals, a classic example being the flattening of fish eyes compared to those of humans, ungulates, birds, and other land-dwelling animals (**Fig. 1A**). Upon deeper probing and the revelation of ever more fine-scale variations in eye structure among species, the question was subsequently extended to microscopic characteristics, such as the number of spectral cone types an animal possesses (Osorio & Vorobyev, 2008; Baden & Osorio, 2019). This number varies widely across the animal kingdom, being two for most mammals, three in primates, four in many fish and amphibians, and soaring (or slithering) to five in birds and reptiles (**Fig. 1B**). These differences are thought to have arisen in response to some form of the selective pressures postulated by Darwin, an assumption which opens the door to the second and third questions animating visual ecology.

The second key question takes a reverse approach to the first, asking: to what extent, and in what ways, are a species' native visual habitat reflected in the form and function of its retina? Investigations seeking to address this question will start outside in the world and work their way backward to relate a species' eye and brain visual circuitry to the environment from which it extracts visual information. This approach, alluded to in the preceding paragraph, is based on a long-standing notion in systems biology which holds that sensory systems have evolved to preferentially extract information of high ecological salience from among the wash of incoming sensory input (Land & Nilsson, 2012; Cronin et al., 2014). In the case of the visual system, the design and performance of the structures responsible for processing these signals will be conditioned primarily on the spectra to which they are exposed (Attneave, 1954; Barlow, 1961;

Maloney, 1986; Ruderman et al., 1998; Simoncelli & Olshausen, 2001; Osorio & Vorobyev, 2008; Lind et al., 2017; Olsson et al., 2018; Zimmerman et al., 2018; Baden & Osorio, 2019). Indeed, the visual systems of a wide variety of vertebrate and invertebrate organisms do appear to be highly tuned to the visual statistics of their natural environment, and in particular to the presence of predator- and prey-like objects (Lettvin et al., 1959; Olberg et al., 2000; Ewert et al., 2001; Simmons et al., 2010; Yilmaz & Meister, 2013; Hoy et al., 2019; Semmelhack et al., 2014; Temizer et al., 2015; Zimmerman et al., 2018). How this is made possible, in terms of retinal organization and dedicated higher-order circuitry allowing rapid processing of biologically relevant information, is an area of active research.

The third question, finally, is an extension of the second. It asks how light content, contrast, and luminance impact structure and function, and attempts to plot an evolutionary path for the latter with reference to those visual stimuli most relevant to an animal's survival and reproductive needs. Building on the earlier example of different animals' photoreceptor complement, many kinds of birds and fish have cones responsive to wavelengths well into the UV range (Baden & Osorio, 2019). One inference that can be drawn from this is that there are short wavelength-biased features of the species' respective niches, such as UV-bright prey (Novales Flaminique, 2012, 2016; Yoshimatsu et al., 2019; reviewed in Losey et al., 1998) or sex- or species-specific color markings (Bennett et al., 1996; Pearn et al., 2001; Smith et al., 2002; Siebeck et al., 2010; Stieb et al., 2017), perception of which was evolutionarily beneficial and which drove the development of UV sensitivity alongside those for lower-frequency wavelengths.

Together, the complementary approaches derived from these questions have driven research in visual ecology since scientists first began to peer into the inner workings of the retina. The field's contemporary story began, as Paley's and Darwin's had a century before, with the eye of the fish (Clarke, 1936; Collin et al., 2009; Luk et al., 2016), and has since expanded to include a wide range of model animals. Classical studies focused on monkeys and cats, which appear to have visual requirements comparable to those of humans in terms of spatiotemporal resolution, spectral sensitivity, and even mental representations and neural coding mechanisms in sensory integration areas (Van Essen et al., 1992; Sereno et al., 1995; Luck et al., 1997; Ungerleider et al., 1998; Van Essen, 2004; Grefkes & Fink, 2005; Elmore et al., 2011; Fize et al., 2011). More recent work has used insects, fish, birds, and rodents to look at how animal eyes, and to a lesser extent higher visual

processing centers within the brain, are adapted to diverse ecological niches, visual needs, and evolutionary histories (Davies et al., 2012; Marshall & Arikawa, 2014; Bostrom et al., 2016; Lind et al., 2017; Baden & Osorio, 2019). The results, as both the intellectual forefathers of this discussion predicted, have revealed an astonishing variety of forms and adaptations.

1.2 Variety in the visual kingdom: Understanding vision in the natural world

The most familiar visual system specialization, and one of the most thoroughly studied, is the *fovea centralis* (**Fig. 2A, D**). Found in simian primates such as *homo sapiens* (Bringmann et al., 2018) as well as certain species of bird, fish, and reptile (Slonacker, 1897; Wood, 1917; Walls, 1947; Duke-Elder, 1958; Querubin et al., 2009; Potier et al., 2017), the human fovea is responsible for high-resolution vision of the center 2° of the visual field, binocular fixation, and depth discrimination (Rapaport & Stone, 1989). Anatomically, it is defined as a temporally located avascular depression in the tissue structure, constituting ~0.5% of the retina by area, composed of a densely packed layer of retinal ganglion cells (RGCs) which are nevertheless removed from its direct vicinity (**Fig. 2C**), a tightly packed, multi-tiered arrangement of cones peaking at the fovea center, and an almost complete absence of rods (**Fig. 2B-C**; Walls, 1942; Polyak, 1957; Potier et al., 2017). In humans and other primates, foveal ‘midget’ RGCs, the majority type in the central retina (Wassle et al., 1990; 1998; Kolb & Marshak, 2003), tend to be innervated by a single, intermediary bipolar cell, which in turn receives input from a single cone, thus relaying signals which are at once clean and highly specific (Polyak, 1941; Ahmad et al., 2003). Axons projecting from RGCs in the fovea make up about half of the fibers in the optic nerve, while the remaining 50% carry information from RGCs from across the other >99% of the retina (Hughes, 1977; Fukuda et al., 1989). Depending on the species of primate, 20–50% of the primary visual cortex is employed in processing foveal input (Chaplin et al., 2013; Provis et al., 2013; Solomon & Rosa, 2014; Bringmann et al., 2018).

Although originally assumed to be a feature only of the primate retina, the basic architectural motif of the fovea in fact appears across a diverse collection of vertebrates, and even to invertebrates, although with intriguing anatomical and functional variations bearing the hallmark of microevolutionary selection. Seabirds, for example, possess an *area centralis* (**Fig. 2E**), a region of acute central vision which is somewhat less clearly bounded or specialized in terms of circuitry

than the fovea (Hayes & Brooke, 1990). Mammals display an assortment of retinal specializations, often differing in even subtler ways, including variations in cellular properties and distributions, but which are nevertheless fovea-like in their overall function (Hebel, 1976; Mowat et al., 2004; Beltran et al., 2014). Rabbits possess a visual streak, an elongated belt sporting a specialized set of RGCs aligned with the horizon rather than being localized within a circular region (**Fig. 2F**; Provis, 1979), while rodents have cell density gradients across their entire retina (Taio & Blakemore, 1976; Dreher et al., 1984); mice, for instance, exploit the differential distribution of RGC types (Bleckert et al., 2013; Zhang et al., 2012) and expression of cone opsins (**Fig. 2G**; AZel et al., 1992; Glosmann & Ahnelt, 1998; Lyubarsky et al., 1999; Haverkamp et al., 2005; Lukats et al., 2005; Baden et al., 2013). Some animals, including kangaroos, cats, and seabirds, have both a visual streak and an *area centralis* (Stone, 1965; Hughes, 1975; Dunlop et al., 1987; Hayes & Brooke, 1990; Ings, 2007), an endowment which allows them to simultaneously view two separate areas of visual space at high resolution.

In each case, the visual system is sculpted by the kind of light it is exposed to in the species' native visual habitat, whether it be sprawling savannah, dense forest vegetation, or high branches and open skies. And although superficial examination would seem to turn up little similarity among them, each specialization is a variation on a repeating theme, making the retina a rich tapestry for which the particular threads woven into its structure are assembled from a grab-bag of biochemical, morphological, and physiological options.

Despite the wealth of research on the forms of visual adaptation, there is a host of animals for which the precise means of encoding visual information remains mysterious. Still others are latecomers to the scientific scene and are currently the focus of active research. Among this latter group is the zebrafish (*Danio rerio*), a small freshwater ray-finned fish native to the flood plains of India, where it is typically found in shallow, slow-moving streams and rice paddies (EngeAZer et al., 2007; Spence et al., 2008; Arunachalam et al., 2013). As is the case for many surface-dwelling teleosts (Neumeyer, 1992; Champ et al., 2016; Baden & Osorio, 2019), zebrafish are tetrachromats, possessing four cone types responsive to light in UV, blue, green, and red wavelengths (Krauss & Neumeyer, 2003; Meier et al., 2018), each of which appears to be associated with different sets of behavior (Orger & Baier, 2005; Yoshimatsu et al., 2019). Despite these intriguing hints of physiological-behavioral correlates, the hunt for visual specializations

within the zebrafish's eye and brain is little more than a decade old. Yet, even in that relatively short period, it has already turned up surprising new insights into how an animal's visual structures reflect the world it inhabits; including, in particular, those features of high ecological relevance like food, potential threats, and other factors impinging on survival and reproductive habits.

1.3 The zebrafish as a model of visual system form, function, and adaptation

First recognized for its current scientific applications in the early 1980s (Streisinger et al., 1981; Walker & Streisinger, 1983; Chakrabani et al., 1985; Grunwald & Streisinger, 1992), the zebrafish has since become a powerful genetic model of vertebrate embryogenesis, development, and disease— including, importantly, in vision research (Fadool & Dowling, 2008). In addition to its genetic accessibility (Mullins & Nusslein-Volhard, 1993; Driever et al., 1994; Solnica-Krezel, 1994) and the high number of gene orthologues shared with humans (Postlethwait et al., 2004; Force et al., 1999; Goldsmith & Jobin, 2012), the biochemistry, histology, tissue structure, and circuitry of the zebrafish eye are highly conserved in comparison to most other vertebrates (**Fig. 3A**; Kolb et al., 2001; Masland, 2001; Meier et al., 2018), as is its formation from skin- and neuro-ectoderm (Branchek & Bremiller, 1984; Raymond et al., 1985; Kljavin, 1987; Larison & Bremiller, 1990; Burrill & Easter, 1994, 1995; Schmitt & Dowling, 1994, 1996, 1999).

Within the zebrafish retina as in those of many other familiar model organisms, RGCs are first to develop, with the little amacrine interneurons differentiating among them and in the inner nuclear layer above (Schmitt & Dowling, 1994, 1999), followed by horizontal interneurons, then the five types of photoreceptors (UV, blue, green, and red cones, and rods) at the backmost layer of the retina (Kljavin, 1987; Raymond et al., 1995). Bipolar cells are the last set of neurons to differentiate, finally slipping into their place between amacrine and horizontal cells by 70 *hpf* (Schmitt & Dowling, 1999), at which point the vertical pathway (PRs to BCs to RGCs) becomes functional (Stuermer, 1988; Schmitt & Dowling, 1999). The only developmental non-uniformity of note is that, in the zebrafish retina, the first stem cells to exit the cell cycle and differentiate into RGCs do so in the ventral rather than the central retina (Burrill & Easter, 1995; Schmitt & Dowling, 1994, 1995). Their progressive differentiation sweeps clockwise through the nasal, dorsal, and finally across the temporal retina (Burrill & Easter, 1995). Similarly, differentiation of

the other neuronal types proceeds from this ventronasal location (Kljavin, 1987; Burrill & Easter, 1995; Raymond et al., 1995; Schmitt & Dowling, 1999).

As RGCs mature, they grow axons which exit the eye and project across the optic chiasm and into the forebrain (Burrill & Easter, 1994, 1995; Schmitt & Dowling, 1999). Here again, where higher visual processing and integration commence, the zebrafish larva differs—this time markedly—from other laboratory models of visual system function. In mammals, including mice, cats, and primates, RGC axons typically innervate the lateral geniculate nucleus (LGN) in the thalamus, which then projects to visual processing centers in the cortex (Goodale & Milner, 2004). By contrast, the larval zebrafish possesses no structure equivalent to the mammalian visual cortex (Grama & Engert, 2012). Instead, RGC axons project to ten contralateral ‘arborization fields’ (AFs): in the pretectum (AF2-9), in the optic tectum (AF10/11), and (AF1) in topologically close areas including the hypothalamus, with the various AFs broadly serving the function of thalamic structures in mammals (Burrill & Easter, 1994) (**Fig. 3B**). For example, the optic tectum has been identified as the zebrafish homolog of the mammalian superior colliculus (Gandhi & Katnani, 2011), and AF7 as potentially being the LGN’s stand-in structure (Schnitzlein, 1962; Semmelhack et al., 2015). Visual information from the AFs, rather than being directed to cortex-like networks for further processing, is sent straight to motor control regions (Gahtan & O’Malley, 2003, 2005; Arrenberg et al., 2009; Kimura et al., 2013; Severin et al., 2014; Thiele et al., 2014; Semmelhack et al., 2015).

Despite this last, and significant, non-homology, the zebrafish offers a number of pragmatic advantages for realizing visual ecology’s goal of uncovering the evolutionary and developmental relationship between visual environment and the cell- and circuit-level hardware which interprets it. First, zebrafish eggs, embryos, and larvae are translucent and thus highly tractable to biological examination. Second, all age groups are easy and inexpensive to take care of. Third, they have short reproductive cycles, reaching sexual maturity in three or four months (Friedrich et al., 2010; Baier & Scott, 2009; Portugues & Engert, 2009; Mclean & Fetcho, 2001). Progression through the stages of larval development is rapid, so that by 3 days post-fertilization (*dpf*) larvae are free-swimming and display a well-studied repertoire of visually guided behaviors (Easter & Nicola, 1996; Avdesh et al., 2010; Preuss et al., 2014; Temizer et al., 2015), including hunting and feeding activity in response to paramecium-like objects, their prey of choice under experimental conditions

(Bianco et al, 2011; Patterson et al., 2013; Trivedi & Bollman, 2013; Semmelhack et al., 2015; Dunn et al., 2016; Bolton et al., 2019).

The fourth and final benefit of employing zebrafish for visual ecological research is the one most relevant to this study, as well as a prime exemplar of the power of this teleost as a research tool. Over the past several years, the basic spatial, temporal, and spectral statistics of the zebrafish's underwater visual world have been modeled (Chiao et al., 2000; Zimmerman et al., 2018), most recently using hyperspectral image data taken from their home waters in India (Zimmerman et al., 2018) (**Fig. 3C-F**). Subsequent research has built on this paradigmatic foundation, continuing to provide clues about how the zebrafish responds to the relative chromaticity of diverging visual information in three broadly-defined fields: its mid-wavelength-dominated view horizon, the region below this midline where long wavelengths predominate, and its short-wavelength-biased upper visual field (Zimmerman et al., 2018; Yoshimatsu et al., 2019). In addition, there are crucial environmental features that cannot be extracted from the statistics of merely static scenes. Instead, these scenes serve as a backdrop against which more variable stimuli, including other organisms, may be perceived (**Fig. 3C**). For example, baby trout feed on the small zooplankton *Daphnia magna*, which scatter light in the UV range and thus appear as UV bright spots (Novales Flamarique, 2012, 2016) against the UV- and blue-shifted upper visual field (Janssen, 1981; Zimmerman et al., 2018) to which they localize via phototaxy (Ringelberg, 1964). It has been suggested based on anatomical, functional, and behavioral evidence (Schmitt & Dowling, 1999; Semmelhack et al., 2015; Zimmerman et al., 2018; Yoshimatsu et al., 2019) that zebrafish larvae do the same for another microorganism, *Paramecium caudata*. Additionally, by hugging the bottom of the pool or riverbed, maturing zebrafish can exploit the brightness gradient in the UV channel to aid in the detection of larger predatory organisms, which will appear as UV dark silhouettes against the relatively bright short-wavelength background above them (Losey et al., 1999; Cronin & Bok, 2016)

1.4 Specializations within the larval zebrafish visual system

Given what we know about zebrafish vision, how does the larva encode the statistical asymmetries present in its visual milieu? How does it extract information about relevant features within its environment, including potential predators and prey, with reference to the sensible-visual backdrop

against which they appear? If specializations are in place for pressing environmental non-uniformities such as these into service for biological advantage, at what level of visual processing (retinal, pretectal, or tectal) do they begin to emerge, and what components of the system do they utilize (anatomy, function, cellular and regional interconnectivity, subcellular characteristics, or some combination thereof)?

Over the past two decades, research in molecular biology, in cell and circuit physiology, and in behavior, exploring these questions from their different perspectives, has gradually converged upon an answer. The consensus emerging from the various lines of enquiry is that the zebrafish retina and visual brain are highly specialized structures, both in terms of architecture and function. Furthermore, among these specializations, one appears to be designed with particular reference to stimuli connotative of prey-like objects, including the paramecia mentioned earlier.

The first wave of studies providing evidence for this was behavioral. Zebrafish are omnivores, ingesting algae, vascular plant material, insects, and zooplankton (McClury et al., 2006; Spence et al., 2007; Parichy, 2015). But the component of their diet which has sparked the most intense research interest is the small aquatic microorganisms present in freshwater systems. When presented with a prey-like stimulus, such as a bright spot of light, larvae commence with a highly stereotypical action sequence (Borla et al., 2002; Gahtan et al., 2005; McElligott & O'Malley, 2005; Hernandez et al., 2002; Bianco et al., 2011; Patterson et al., 2013; Trivedi & Bollman, 2011; Semmelhack et al., 2015; Mearns et al., 2019). In brief, larvae approach their prey from slightly below, propelling themselves forward in short spurts with small, rapid tail oscillations and orienting themselves by means of long-duration tail deflections to one or the other side (referred to as 'forward swims' and 'j-turns', respectively) (Borla et al., 2002; Bianco et al., 2011; Semmelhack et al., 2015). At the same time, they converge their eyes so as to create a binocular visual field with which to view the prey-object (Bianco et al., 2011; Patterson et al., 2013; Semmelhack et al., 2015). Eye convergence always either precedes or is concurrent with, but never follows, the tail movements associated with hunting behavior, and this vergence is maintained throughout the entire period during which the larvae track their prey (Bianco et al., 2011). Importantly, by positioning themselves so as to place the prey-object above and to the front of the midline of their field of binocular vision (Mearns et al., 2019), the fish ensure that the image falls on cells located in the ventrotemporal retina.

As early as 1999, the temporal retina of zebrafish larvae had been shown to possess characteristics which distinguished it from the rest of the retina. These included a high density of tightly packed, tiered cone photoreceptors and a relative paucity of rods (Schmitt & Dowling, 1999), reminiscent of regions responsible for high acuity vision in other animals, including the primate fovea, the cat *area centralis*, and the *area temporalis* present in many teleosts (Walls, 1942; Ito & Murakami, 1984; Robinson, 1987; Collin & Pettigrew, 1988a, b; Zimmerman et al., 1988; LaVail et al., 1991; Henrickson, 1995). Subsequent work revealed that, at 7 *dpf*, the distributions of all photoreceptor types varied systematically to match spectral trends in the environment as well as specific behavioral needs, and that, in particular, UV cone density peaked below the horizon, reaching $\sim 35,000/\text{mm}^2$ in the *area temporalis* (Zimmerman et al., 2018). Building on these findings and taking into account the spatial, temporal, and spectral statistics of natural scenes (Baden et al., 2019; Nevala & Baden, 2019) as well as the zebrafish larvae's tendency of positioning themselves so as to view their prey with the ventrotemporal retina, recent studies have hypothesized that this region should prioritize UV-ON circuits driven by prey capture. Large-scale recordings of photoreceptor and bipolar cell activity have confirmed this notion, while simultaneously demonstrating dramatic differences in chromatic processing across the eye as a whole (Zimmerman et al., 2018; Yoshimatsu et al., 2019). Prey identification begins, then, not with visual centers in the brain, but in the retina itself, with the very first layers of light-processing cells.

The neural circuits used to transmit prey information from eye to brain have also been partially mapped. By tracing RGC axons innervating AF7 and the optic tectum, the two AFs which activate in response to prey stimuli, back to their cell bodies in the retina, researchers have determined that it is only a very specific subset of RGCs which appear to be responsible for most, if not all, of the signal (Semmelhack et al., 2015). These may consist of as few as two RGC types from the more than 50 which have been structurally classified: B2 RGCs with bistratified dendritic arbors, and D1 RGCs with diffuse ones (for sample images, see **Chapter 4: Figure 1B-D**). Consistent with this data, it has been shown that, when their somata are located in the temporal retina, both types project axons preferentially ($\sim 95\%$) innervate AF7 and to a lesser degree the OT's stratum opticum. Conversely, B2 and D1 RGC somata positioned elsewhere within the ganglion cell layer (GCL) are more uniformly distributed than those within the ventrotemporal area, and have postsynaptic targets in areas other than AF7. In fact, AF7 is the only AF receiving the majority of its innervation from the temporal retina (Robles et al., 2014).

Thus, the retina of the larval zebrafish, along with many associated brain structures, is highly anisotropic, with different areas varying both in terms of anatomical features and functional properties. Nevertheless, the *area temporalis*, dubbed the ‘acute zone’ (abbreviated ‘AZ’), is still considered to be something of a first among equals, garnering interest not only for the extent to which it is specialized– the cellular real estate and metabolic resources allocated to it– but for the singular purpose toward which that functional design appears to be suited: prey capture.

1.5 The new frontier: Eyeing the zebrafish inner retina

There is strong evidence, then, not only for the presence of specializations within the larval zebrafish retina, but for the existence of a dedicated prey-detection system present within the ventrotemporal area. Functional data from photoreceptors and bipolar cells indicate that this circuitry is strongly UV-dependent (Zimmerman et al., 2018; Yoshimatsu et al., 2019), while anatomical evidence points to the acute zone as being a structurally defined feature of both retinal and pretectal circuits clearly differentiated from neighboring visual processing regions (Robles et al., 2014; Semmelhack et al., 2015). However, little is known about how RGCs, the eye’s sole information output channel, are structurally and functionally tuned to process prey-like features of the visual environment; and, critically, if and how these features differ between RGCs in the acute zone and those surveying other parts of the visual field.

In this doctoral project, we use a combination of confocal and *in vivo* 2-photon imaging, behavioral tests, and computational analysis to profile acute zone RGCs and compare their features with those of ganglion cells in other regions of the retina. Our experimental approach was guided by six objectives which build consecutively upon one another to construct a complete picture of inner retinal form and function in the AZ, and the particular behavioral output with which AZ activity correlates. The results corresponding to each of these aims are addressed individually in the following chapters. In chapter 2, we characterize the densities of inner retinal somata across the eye’s hemisphere, as well as structural and metabolic differences associated with different retinal regions. Taken together, these preliminary anatomical investigations provide clues which collectively point to the possibility of nonhomogeneous GCL physiology and circuit connectivity. Chapter 3 then describes the transgenic line we generated to selectively probe RGC function and cytoarchitecture, and our characterization of the promoter/reporter system. In chapter 4, we use

this transgenic line to build on the grosser anatomical non-homogeneities presented in the second chapter, comparing the distribution of individual RGC types between the acute zone and non-adjacent regions of the retina, and attempting to determine the morphological identity of those types as well as any region-specific differences among them. We then go on to profile the morphologically defined RGC types within different areas of the retina according to the distributions of their synaptic densities. In chapter 5, we map the functional organization of acute zone RGCs, and ascertain their response kinetics, polarities, and chromatic preferences, using full-field tetrachromatic light stimuli. In chapter 6, we analyze larval behavior in response to a range of spatial stimuli designed with reference to the AZ RGC response profile described in chapter 5, comparing the fish's motor output when it is presented with prey-like and non-prey-like light stimuli. Finally, in chapter 7, we draw together the threads of discovery presented in the preceding chapters, discuss their implications, and offer suggestions for directions which future research might take to build on our findings.

In summary, we show that the function, distribution, and many aspects of morphology among acute zone RGCs differ from those of cells positioned in the nasal, dorsal, and ventral retina, with the most marked differences existing between nasal and acute zone RGCs. We further describe the outcomes of preliminary research aimed at matching the responses of acute zone RGCs to prey-like stimuli associated with prey detection, hunting, and capture behavior. These cellular characteristics reproduce the structural and functional biases found among outer-retinal cells and extend the systematic processing of visual statistics to the innermost retinal layers, while the broad behavioral paradigms with which they correlate conform to the dedicated UV prey capture system proposed by earlier studies.

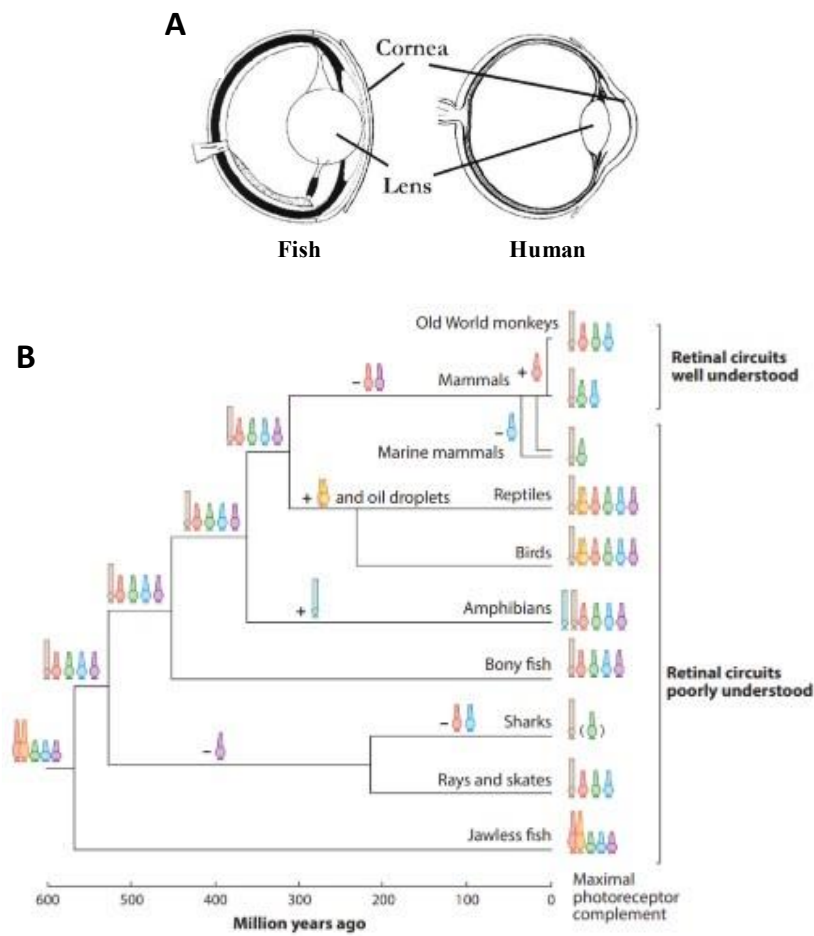


Figure 1: Homologies in the vertebrate eye.

(A) Schematic of fish and human eye. **(B)** Photoreceptor lineages, including cone and rod complements in a range of model organisms. (Adapted from Baden & Osorio, 2019).

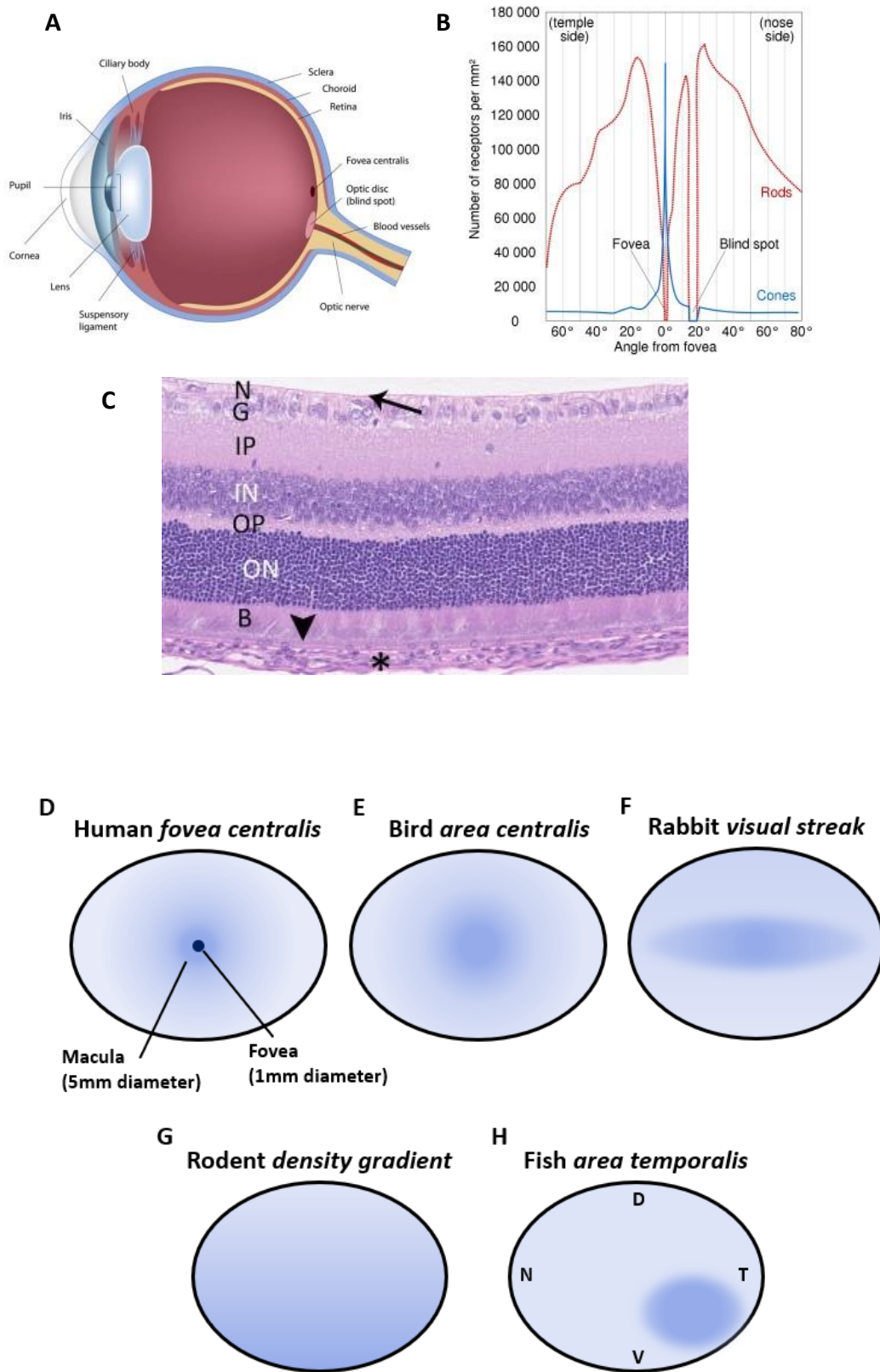


Figure 2: Different distributions of retinal neurons create the variety of specializations with which vertebrates see.

(A) Schematic of human eye. (B) Distribution of rods and cones as quantified along a line drawn passing through the fovea and blind spot of the human eye. (C) Tissue structure of human eye. Note that the absence of RGCs in front of the foveal PRs does not reflect a paucity of ganglion cells responsible for carrying light signals from this region, but that the relevant inner retinal cells responsible for foveal signals are located along the extremities of the fovea. See list of abbreviations for labels of respective layers. (E-H) Representative examples of the primate fovea (E), the area centralis found in birds and other animals (F), the visual streak (G), the cellular density gradients characteristic of the retinas of mice and other rodents (H), and the area temporalis, a generic form of which is found in many species of fish (I). N, nasal; D, dorsal; T, temporal; V, ventral.

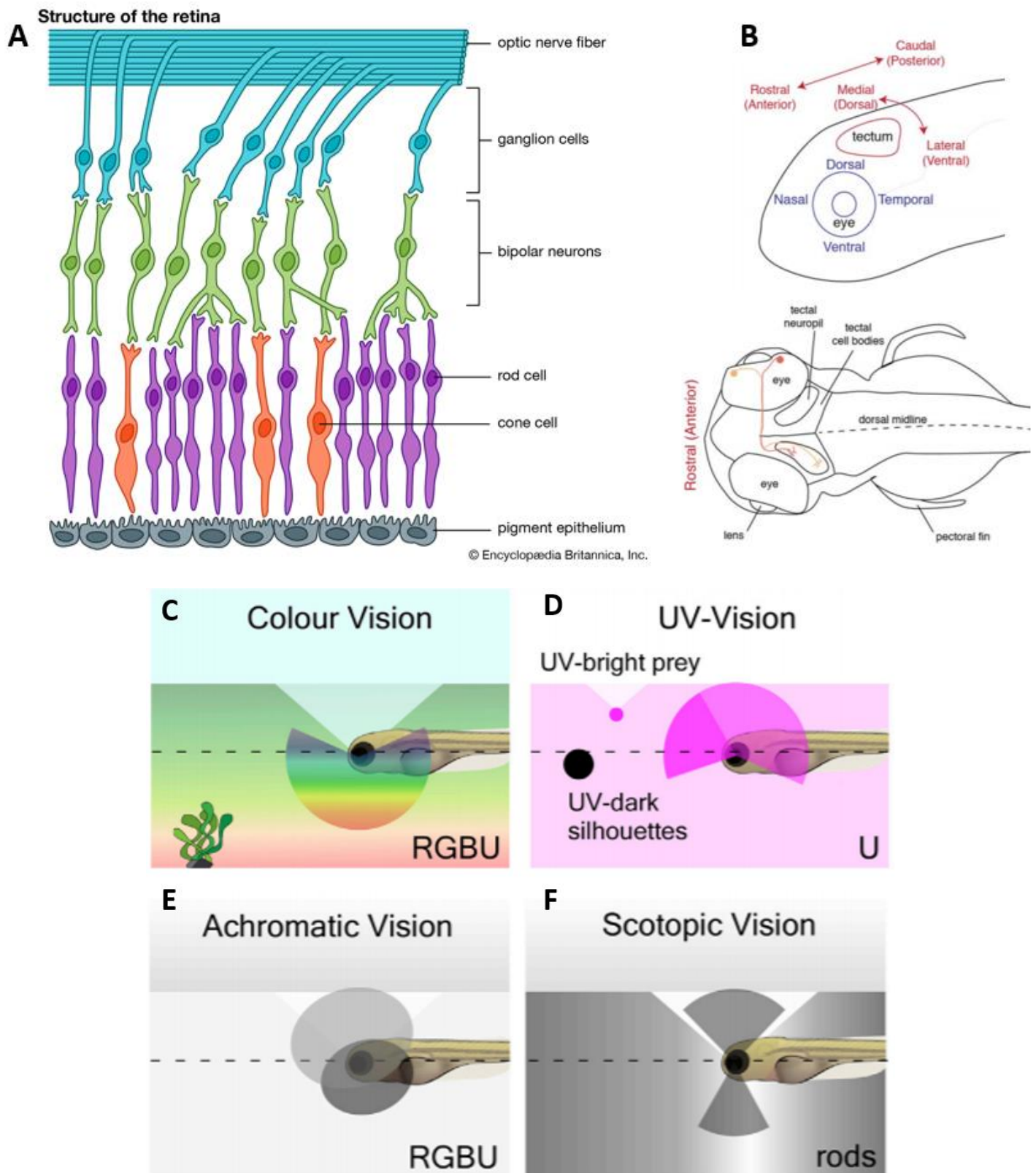


Figure 3: The zebrafish larva's retina and visual-processing brain structures process information from a highly complex visual world. (A) Schematic of human retina. The zebrafish retinal neuronal complement and circuit structure are similar, except that their cone population consists of one additional type, UV cones. **(B)** The retinotectal connection in zebrafish. The lateral view (left panel) orients the eye and tectum with respect to the larva's head. The pretectal AFs are located just below the tectum. The dorsal view (right panel) shows a sample retinotectal topographic map. **(C-F)** Retinal circuits for color vision **(C)**, UV(B)-monochromatic vision **(D)**, achromatic vision **(E)**, and scotopic vision **(F)** are biased to different parts of the visual field, dependent on the spectral profile and

2. ANISOTROPIC CELL DISTRIBUTIONS *and* RESOURCE DIFFERENTIALS WITHIN THE INNER RETINA

2.1 Background

Unlike the 7 *dpf* larva's differential distributions of rods and cone types (Schmitt & Dowling, 1999; Yoshimatsu et al., 2019), adult zebrafish feature a crystalline pattern of photoreceptors, with red, green, blue, and UV cone densities of 2:2:1:1 invariant across the retina (Allison et al., 2010; Engstrom, 2010; Salbreux et al., 2012). It was long assumed, for the most part tacitly, that the same mosaicy held broadly for retinal cells in the inner nuclear layers as well, both during development and in adulthood, so that by uniformly tiling the retina each type evenly sampled visual space as they did in other vertebrate models (Sun et al., 2002; Wassle et al., 2004; Volgyi et al., 2005). However, this view was challenged by the discovery that RGC types in the adult were irregularly arranged across the retina, with no apparent ordering either between or within individual morphologically defined types, and that, further, the density of all RGCs increased roughly threefold moving dorsonasal to ventrotemporal (Mangrum et al., 2002). Thus, moving from outer to inner retinal cell layers, the adult retina appears to lose the systematic placement of its cell types, becoming more cryptically ordered.

In contrast to adults, the recent characterization of the anisotropies present among the photoreceptor (Yoshimatsu et al., 2019) and bipolar cell complements of larvae (Zimmerman et al., 2018) suggests that at no stage of visual processing do cells serve as mere pixel-detectors, but that light signals begin to diverge in a systematic manner among the first light-responsive cells. Presumably, the larva's innermost retinal layers, consisting of ganglion and amacrine cells, will reflect the anatomical specializations present in the outer layers to preserve and diversify the information channels for which they are responsible. Presumably, too, any density biases among inner retinal neurons will be matched with additional structural, functional, and metabolic resources, most likely by means of the Muller glia cells (MGCs) whose end processes contact RGCs (Poitry-Yamate & Tsacopoulos, 1991; Pfeiffer et al., 1994, 1995; Reichenbach & Robinson, 1995; Newman & Reichenbach, 1996; Reichenbach & Bringman, 2013) and by retinal mitochondria distributed among neurons and glia (Country, 2017; Damsgaard et al., 2019). However, little is known about either RGCs or displaced amacrine cells (dACs) within the GCL, or about the amacrine cells (ACs) and MGCs whose somata are dispersed throughout the INL.

How these cell populations are organized within their respective layers, and the extent to which each type's distribution complements those of the other types, remain to be explored. On a subcellular level, too, the distribution of ATP-producing mitochondria within inner retinal cell types is unknown.

The question which remains to be addressed, then, is whether the outer retina's structural anisotropies extend into the inner retina? And if so, which of its neuronal and glial populations demonstrate region-specific variations? To investigate whether and how RGCs, dACs, INL ACs, and MGCs are regionally specialized, we performed cell counts of all four cell types, obtaining density maps of their relative number and distributions across the 3D retinal hemisphere. We show that the densities of all four cell types vary across the eye, with the highest number of RGC, dAC, and MGC somata clustered within the ventrotemporal retina. Additionally, we identified other structural characteristics of inner retina, with regard to MGCs and mitochondrial distributions, which provide clues as to how the eye provides for the different structural and metabolic demands created by the anisotropic neuronal distributions.

2.2 Materials and methods

Animal care and lines. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1968 and approved by the animal welfare committee of the University of Sussex. Adult animals were housed under a standard 14/10 light/dark cycle and fed 3 times daily. Larvae were grown in E2 solution (1.5M NaCl, 50mM KCl, 100mM MgSO₄, 15mM KH₂PO₄, 5mM Na₂HPO₄) and treated with 200 μ M 1-phenyl-2-thiourea (PTU; Sigma) from 12 *hpf* to prevent melanogenesis (karlsson et al, 2001).

For all experiments, we used 6-8 *dpf* zebrafish larvae. Since gonadal differentiation has not yet occurred at this stage of development, representatives of either sex were used indiscriminately. In addition to non-transgenic nacre (Lister et al., 1999), roy (D'Agati et al., 2017) and casper (White et al., 2008) larvae, the genetically-modified fluorescent reporter lines *Tg(Ptf1a:dsRed)* (Lin et al., 2004; Jusuf & Harris, 2009) and *Tg(GFAP:GFP)* (Bernardos & Raymond, 2006) were used.

Tissue preparation, immunolabeling, and imaging. For immunohistochemistry, larvae were culled by tricaine overdose (800 mg/l) and fixed in 4% paraformaldehyde for 28 minutes at room

temperature before being washed in 1 M Calcium-negative phosphate-buffered saline (PBS, pH 7.4: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ diluted in d₂H₂).

Retinae were incubated in permeabilization/blocking buffer (PBS with 0.5% Triton X-100 and 5% normal donkey serum) at 4°C for 24 hours, after which they were transferred to the appropriate labeling solution (#1, #2, or #3). **Solution #1:** For nuclear labeling, tissue was incubated at 4°C in blocking solution with Hoescht 33342 nuclear dye (Sigma, H21492, 1:2000) for 24 hours. **Solution #2:** For membrane staining, tissue was incubated at 4°C in blocking solution with bodipy membrane dye (Sigma, D3821, 1:1000) for 24 hours. **Solution #3:** For immunostaining, tissue was incubated at 4°C for 72 hours in primary antibody solution (chicken anti-GFP (AbCam or 13970, 1:500), rabbit anti-cox iv (AbCam, 16056, 1:500), diluted in permeabilization/blocking solution). Samples were rinsed three times in PBS with 0.5% Triton X-100 to remove unattached primary antibodies, then transferred to secondary antibody solution (donkey anti-chicken IgG CF488 A conjugate (Sigma, 1:500), donkey anti-rabbit IgG CF568 conjugate (Sigma, 1:500), diluted in permeabilization/blocking solution) and incubated at 4°C for 24 hours. Finally, samples incubated in solutions #1, #2, and #3 were rinsed three times in PBS with 0.5% Triton X-100 to remove excess dye/unattached secondary antibodies before being mounted in mounting media (VectaShield, Vector, H-1000) for fluorescent imaging.

Confocal stacks and individual images were taken on Leica TCS SP8 using 40x water-immersion objective at xy resolution of 2048x2048 pixels (pixel width: 0.162 µm). Voxel depth of stacks was taken at z-step 0.3-5 µm. Contrast and brightness were adjusted in FIJI (NIH).

Cell density mapping. GCL nuclei (stained with Hoescht 3342), dAC and AC (*tg(Ptf1a:dsRed)*), and MGC (*tg(GFAP:GFP)*) (immunolabeled against GFP) somata were quantified in Fiji from confocal image stacks of whole eyes. These image stacks, now updated with the approximate 3D locations of all cell bodies from each respective retinal type, were then converted to 2D fisheye projections using custom-written scripts in Igor Pro 6.37 (Wavemetrics). Density maps representing forward-to-back views of the retinal hemispheres were generated by extracting the total number of somata within a 28 µm radius of each individual soma.

RGC density estimation across the visual field. To project the determined RGC distribution into visual space, we measured the mean size of the larval retina to be 300 µm in diameter, and assumed: first, that both the eye and lens follow an approximately spherical curvature around a

center point; second, that any given RGC receives light-induced signals from cones, which themselves collect light from a point in space aligning with a straight line connecting any given cone to the visual world through the center of the lens; third, that the RGC densities in the left and right retina are mirror reflections of one another. Using these as parameters, RGC density was projected into a sinusoidal map of visual space (Yoshimatsu et al., 2019) to recreate the RGC receptive field locations across the full monocular visual field, from which we extrapolated binocular visual fields: one representing the larvae's eyes when they are in a resting, non-converged position prior to prey detection, and one corresponding to the post-converged state for prey localization subsequent to detection.

Image processing and quantification. Quantifications were made using the Analyze Particle tool provided in FIJI. For comparison of GFAP mean fluorescent intensity, region of interests (ROIs) were drawn manually around MGC processes in the GCL of the ventral, nasal, dorsal, and AZ. For mean area of MGC endfeet in the GCL of the same four regions, images were converted to 8-bit binary, background subtracted (RBR: 100 pixels), Gaussian blurred (1.00 Sigma radius), and thresholded. For *cox-iv* punctae quantifications, ROIs were drawn to include all punctae in either the IPL or the GCL of the ventral, nasal, dorsal, and AZ, and the quantity and size of punctae extracted in the same manner as for MGC endfeet. FIJI data was organized and bar graphs representing each data set were generated using Excel.

Statistical analysis. Statistical analysis of data obtained using FIJI was performed with InStat (GraphPad, San Diego, CA). For all IF data sets, the non-parametric Mann-Whitney test (unpaired t-test) was applied to generate a two-tail P value. Number of ROIs (used for mean fluorescent intensity measurements) and MGC endfeet (used for endfeet size) from each retinal region compared is provided in accompanying respective figures.

2.3 Results

Anisotropic distributions of RGCs, dACs, and ACs. To study the distributions of the larva's RGC, dAC, and AC complements, we fluorescently labeled the different cell populations. For dACs and ACs, we expressed mCherry under the retinal interneuron-specific nuclear marker *Ptf1a* (Jusuf & Harris, 2009). Since no antibody, cell-molecular stain, or line expressing a fluorescent

marker is available which selectively and universally labels RGCs at 7 *dpf*, we instead calculated their distribution by staining cell nuclei and subtracting genetically defined dACs within the GCL from this total. We projected each 3D stack comprising the retinal hemisphere into a distance-preserving 2D plane, recreating the respective cell populations as density maps reflecting the number of somata present in each retinal volume to which a given 2D region corresponded (**Fig 1A-C**).

Of the ~5,750 cells in the GCL, ~765 were dACs, with the remainder comprised of RGCs and a small fraction of glial cells (**Fig. 1B₁, B₂, C₁**). The distributions of both major GCL populations were anisotropic, with densities peaking in the ventrotemporal and nasal areas. As had been implicated by the results of dye tracing experiments in adults, the 7 *dpf* larva's RGC complement (~4985 cells in total) displayed a more than two-fold increase in density within the ventrotemporal retina compared to that of the dorsal, ventral, or retinal apex, with the AZ comprising ~400 RGCs. A second, smaller RGC peak (~1.2-1.3x) occurred in the nasal area (**Fig. 1C₁**). Similarly, dACs (~765 cells in total) peaked in the nasal and AZ, although these density hotspots were more smeared out than for those of RGCs (**Fig. 1B₁**). The higher cell density at the leading edge of the eye appears to correspond to the ciliary marginal zone (CMZ), a germinal band where smaller, more tightly-packed differentiating cells are still in the process of moving into their final positions (John, 1977; Wan et al., 2016). Incidentally, this rapid differentiation process, in which even a few hours likely contribute a substantial cell count difference, may partly explain the 1.5-fold increase over the 4,000 RGCs estimated by Robles and colleagues (2014).

By contrast, amacrine cells in the INL (~3069 cells in total) exhibited a much more variable distribution (**Fig. 1B₃**). Significantly, the density hotspots characterizing RGCs and dACs in the nasal and acute zone were absent. Instead, these regions featured a lower number of AC somata than in other regions, while the areas of comparatively high cell density were the ventral and retinal apex. Together with dACs, the total AC population in the GCL and INL was distributed homogeneously across the eyecup (**Fig. 1C₂**). As a result, the AZ had a low AC-to-RGC ratio (~3:8) in comparison to the retinal mean (~6:8). The main exception was the CMZ; here, the highest densities occurred along the leading edge of the dorso-temporal retina, although whether this is a specifically developmental feature, or the fact that it is represented primarily in one quadrant of the INL indicates a functional significance, remains unclear.

Prior to prey detection and eye convergence, there is little overlap between the larva's 170° field-of-view eyes, with the total visual field being mostly monocular (Zimmerman et al, 2018). During hunting, ocular vergence increases from 36° to 76° (Bianco et al., 2011; Patterson et al., 2013; Trivedi & Bollman, 2013), with the frontal binocular overlap increasing from 26° to 66° (Yoshimatsu et al., 2019) at the expense of decreasing the eyes' total spatial detection limit, primarily within the rearward-facing visual field (**Fig. 1D-G**). Based on these numbers, we computed the binocular visual field for GCL cells when the larva's eyes are in the non-converged resting position and the converged state following prey detection. When projected into binocular visual space, with eyes unconverged, the RGC and dAC AZ hotspots aligned with the upper-frontal visual field of either eye (**Fig. 1D, E**), the areas in which prey-like stimuli are known to be most effective in eliciting prey capture sequences (Bianco et al., 2011; Mearns et al., 2019). Upon convergence, the monocular view fields superimposed along the central length of binocular visual space, creating a region of high acuity in the frontal visual field. This acute zone reached a peak at ~30° above the horizon, (**Fig. 1F, G**) where the UV-signal from microorganisms is most prominent (Yoshimatsu et al., 2019) and in which prey-like stimuli are placed by body and fixational eye movements in preparation for strikes (Sammelhack et al., 2014; Antinucci et al., 2019; Mearns et al., 2019).

Muller glia distribution and region-specific structural support. In order to map Muller glia distributions, we expressed GFP under the glial cell-specific marker GFAP and created a 2D projection map of the location of MGC somata as above. The ~887 MG somata in the INL were for the most part uniformly distributed, the only exceptions being small hotspots in the nasal and temporal retinas. In the temporal retina, the region of highest density occurred at the midline, while a second, lower-density region below it overlapped with the RGC/dAC hotspot corresponding to the acute zone. The germinal zone at the eye's leading edge was almost entirely absent, in line with previous reports that MGC cells have for the most part finished migrating basally into the INL and attained a mature morphology by 72 hours post fertilization (*'hpf'*; Rapaport et al., 2004; MacDonald et al., 2015).

GFAP is an intermediate filament protein (Parry & Steinert, 1999) diffusely localized in the cytoplasm of astrocytes (Jacque et al., 1978; Eng et al., 2000), including MGCs in the retina (Guerin et al., 1990; Vaughan et al., 1990; Lewis et al., 1992). In addition to allowing us to verify

the identity of MGCs by their characteristic elongated morphology extending from the top of the PR layer to RGC somata (Hollander et al., 1991), other aspects of their overall structure could be observed. In particular, we noticed increased fluorescence in the ventrotemporal retina in and around the acute zone's RGC/dAC hotspot, indicating that there is a greater mass of MGC end processes in this area (**Fig. 2A-G**). Undoubtedly, the increased number of temporally located MGC cells contributes to this effect. However, given that the MGC density increases only ~1.5-fold moving nasal to temporal, while the fluorescent signal increased by a factor of 3 (**Fig. 2G**), it appears also to be the result of increased surface area of individual end processes (**Fig. 2F**). Thus, the structural integrity of the relatively cell-dense acute zone appears to be supported by both an increase in the number of MGCs in the temporal retina, as well as differences in the gross structural features of the MGCs themselves.

Mitochondrial allocation. In addition to requiring increased structural support, regions of relatively high cell density will likely also be more energetically demanding than more sparsely populated areas. These elevated functional and metabolic demands would be predicted to increase consumption of both oxygen and glucose for ATP synthesis. One indirect means of measuring relative consumption of ATP-precursor molecules is to determine the number of supporting glia in these areas, which shuttle nutrients from the bloodstream and redistribute them throughout the retina (Reichenbach & Bringmann, 2013) and serve as generators of ATP through glycolysis and oxidative phosphorylation (Country, 2017). Another method, particularly useful for studying the minority of vertebrate retinas which are vascularized by intra- or pre-retinal vessels (Country, 2017), is to measure the mitochondrial allocation within regions of varying cell density (Germer et al., 1998; Yoshimatsu et al., 2019). In the vascular retina of zebrafish larvae, which receives oxygenated blood from both the choroid rete mirabile behind the RPE and a pre-retinal capillary bed lining the surface of the retina (Alvarez et al., 2007; Cao et al., 2008; Kaufman et al., 2015), mitochondria are not restricted to PR inner segments as they are in vertebrates with avascular retinas (Bentmann et al., 2005; Country, 2017). Instead, the availability of oxygen to the inner retina correlates with the presence of these organelles in the plexiforme and ganglion cell layers (Kageyama & Wong-Riley, 1984; Bentmann et al., 2005), providing a window into energy production by oxidative pathways.

To gain insight into how differences in energy requirements across the GCL might be met by means of differential mitochondrial distribution, we performed immunostaining against the cox iv antigen, a subunit of the cytochrome c oxidase hetero-oligomeric enzyme located in the inner mitochondrial membrane (Li et al., 2006). Our labeling revealed two characteristics of mitochondrial localization in the retina. First, MGCS contain mitochondria throughout the entire length of their cytoplasmic space (**Fig. 3A**), similar to MGCs in vascularized mammalian retinas (Germer et al., 1998). This suggests that the relatively higher number of mitochondria-dense MGCs in the temporal retina allow them to provide additional energetic as well as structural support in this region. Second, the IPL featured a mitochondria-rich band in the ON-layer of the AZ, and to a lesser extent as well in the nasal region (**Fig. 3A-C**). By contrast, in the ventral and dorsal retina, the mitochondrial punctae were diffusely localized throughout the IPL, exhibiting neither any apparent order nor any increase in the relative density between ON and OFF layers.

In addition to this region-specific distribution pattern, the density of punctae within the four (N, D, AZ, V) regions of the IPL varied in more subtle ways. First, while the number of punctae within both plexiform and cell body layers were remarkably similar between nasal and AZ, both retinal quadrants were more populated by cox-iv punctae than either ventral or dorsal (**Supplementary Fig. 3F, H, I, K**). This effect was not due to the narrowing of IPL and GCL in both retinal extrema, but rather to an increase in the number of cox-v punctae distributed across the IPL and GCL of nasal and AZ (**Supplementary Fig. 3F, I**). Nevertheless, the same fraction of total area across all four areas was inhabited by cox-iv punctae (**Supplementary Fig. 3H, K**). This appeared to be the effect of increased punctae size within dorsal and ventral, such both possessed relatively fewer, but larger, punctae than did either nasal or AZ, which correspondingly had more numerous, smaller punctae (**Supplementary Fig. 3G, J**). Thus, while mitochondrial content does not appear to vary across the inner retina, it appears that the RGC and dAC hotspots in the nasal and acute zone are matched not only by restrictions on the distribution of mitochondria within the IPL, but potentially also by the organelles' relative spread within IPL and GCL.

2.4 Discussion

Previous studies have shown that the major neuronal cell types in the larval ONL and INL are arranged for efficient coding of visual field statistics and behaviorally relevant stimuli

(Zimmerman et al., 2018; Yoshimatsu et al., 2019, 2021). In particular, these retinal layers feature a pronounced acute zone in the *area temporalis* with a preponderance of UV-ON circuits (Schmitt & Dowling, 1999; Zimmerman et al., 2018; Yoshimatsu et al., 2019), which functions in combination with a set of well-characterized fixational eye movements to facilitate visually-guided prey capture (Semmelhack et al., 2014; Antinucci et al., 2019; Mearns et al., 2019; Yoshimatsu et al., 2019). In this chapter, we have provided evidence that this organizational principle extends to the innermost retinal cell layers, both neuronal and non-neuronal, with the density of each of the four cell types surveyed varying across the retinal hemisphere.

As predicted from the work on photoreceptors (Zimmerman et al., 2018; Yoshimatsu et al., 2019), the density of RGCs was elevated within the AZ (**Fig. 1C₁**). In combination with the homogeneous distribution of GCL and INL ACs, this served to create a low AC:RGC ratio within the ventrotemporal retina. This apparent reduction of inhibitory circuits bears a striking resemblance to the relatively low expression of genes involved in GABAergic neurotransmission (Peng et al., 2019) and reduced inhibitory tone in the primate fovea (Crook et al., 2011; Sinha et al., 2018), which suggests either a paucity of GABAergic ACs or the absence of one or more types. Since larval zebrafish need to process the signal from a small number of cones for visual prey detection (Yoshimatsu et al., 2019), this effect might serve to boost signal-to-noise computations by relieving inhibitory burden, the function it is thought to serve in the primate's low-convergence foveal circuit (Baden et al., 2019).

Whatever the effect of reduced inhibition, the relative density increases of RGCs and dACs in the AZ help confirm the notion that this is a key visual information highway requiring increased processing capacity. What signals the AZ's channels encode, their quantity and diversity relative to other retinal regions, and how many are unique to the ventrotemporal retina remain open questions for both the RGC and AC populations, although the results from PR and BC recordings have provided clues as to the upstream signals feeding into inner retinal circuits (Zimmerman et al., 2018; Yoshimatsu et al., 2019). Another significant question remaining to be addressed with regard to both inner retinal neuronal types are the finer-scale structural anisotropies, at the subcellular rather than merely tissue level, which should be relatable to cell and circuit physiology as they are for PRs and BCs (Robles et al., 2014; Zimmerman et al., 2018; Yoshimatsu et al., 2019). If this is the case, it would provide a complementary, and convergent, approach to

determining the AZ response profile and how this might vary by comparison to other areas of the retina. We explore the possibility of cytoarchitectural anisotropies across different retinal regions in chapter 4.

Paralleling the extensive neural hardware within the AZ, the increased metabolic capacity and structural support from MGCs in this area similarly reflect demands on structural and functional stability which differ across the eye. Whether either function is prioritized within the AZ was not addressed by this study, although the requirement for prey-related signaling suggests that the increased MGC presence cannot be accounted for solely by the high density of neuronal cells in this part of the retina but may be driven by sustained and elevated levels of activation; that is, they are not in place simply in a supporting role but to provide energy for the light responses. This conclusion, as well as the inference from PR and BC data that the anticipated energy expenditure is attributable to ON cells (Zimmerman et al., 2018; Yoshimatsu et al., 2019), is backed up by the differential allocation of mitochondria within the IPL. Although not the only means of recycling ATP precursors which the retina has at its disposal (Country, 2017), it provides the most efficient means of sustaining activity in highly active ON cells in vascularized tissue, and is a feature common to the retina of many primate species (Kageyama & Wong-Riley, 1984).

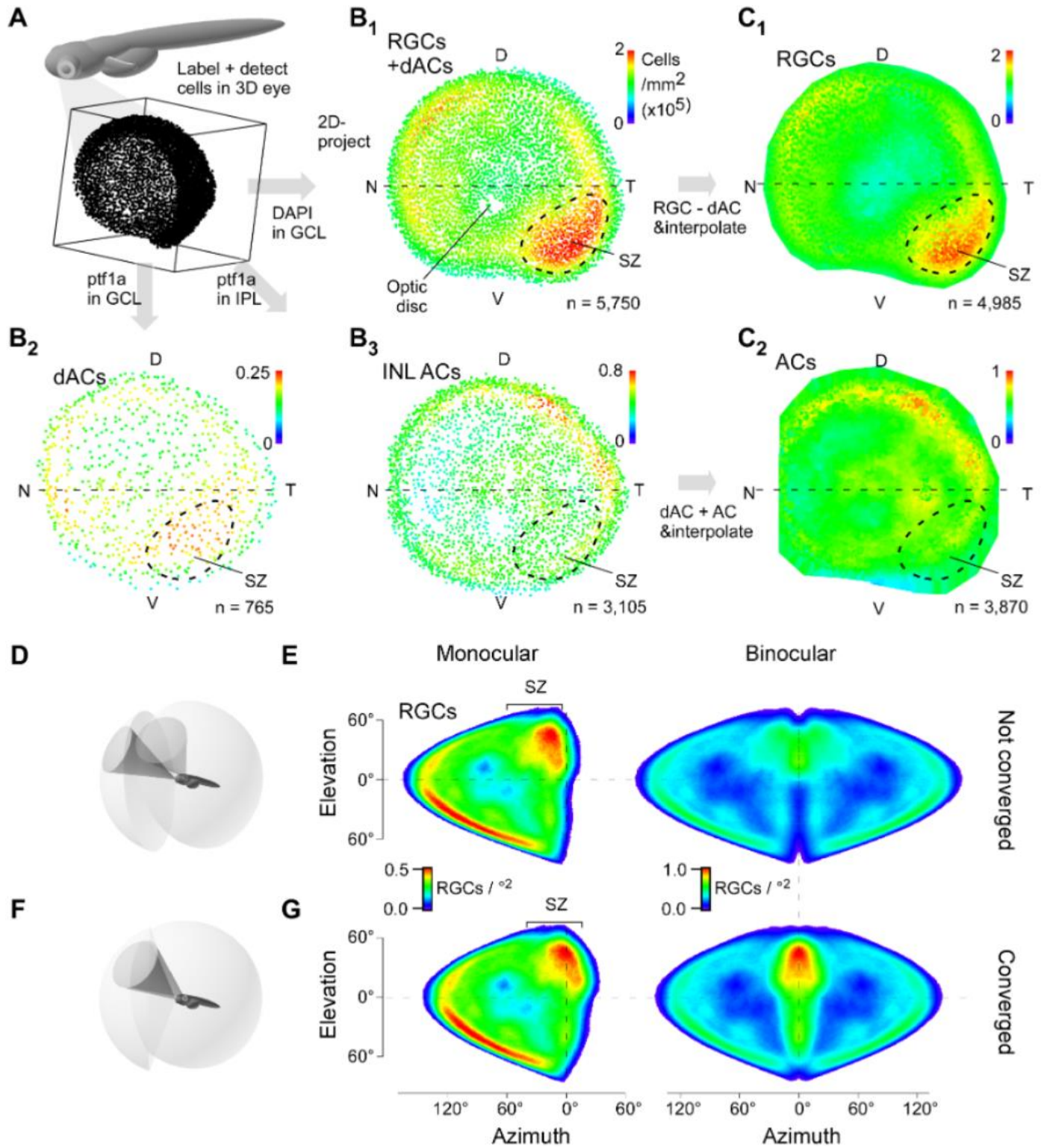
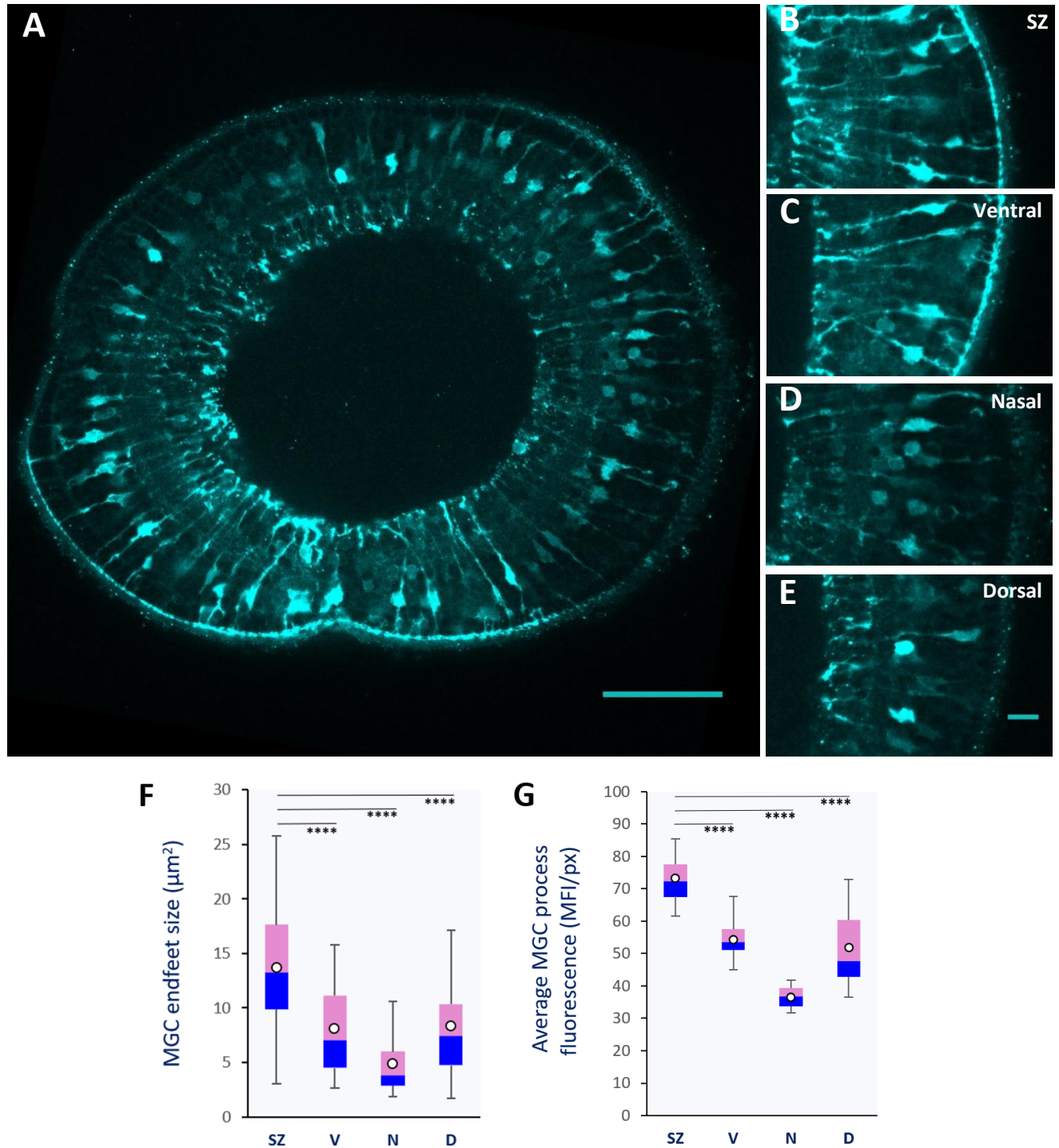


Figure 1: A fovea-like low AC:RGC ratio in the acute zone.

(A) Schematic of larval zebrafish and enlarged 3D representation of the retinal hemisphere's GCL nuclei complement. (B₁₋₃) 2D projections as density maps of detected soma positions of: all GCL cells based on nuclear stain, including RGCs, dACs, and a small number of glia (B₁); all dACs within the GCL based on Ptf1a labeling (B₂), and; all ACs within the INL based on Ptf1a labelling (B₃). (C₁₋₂) 2D projections as density maps of all RGCs (C₁) and GCL/INL ACs (C₂) computed from cell counts in (B). T, temporal; SZ, acute zone; V, ventral; N, nasal; D, Dorsal. (D-G) 3D schematics of non-converged (D) and converged (E) eye positions, and corresponding projections of RGC densities into monocular (F) and binocular (G) visual space.



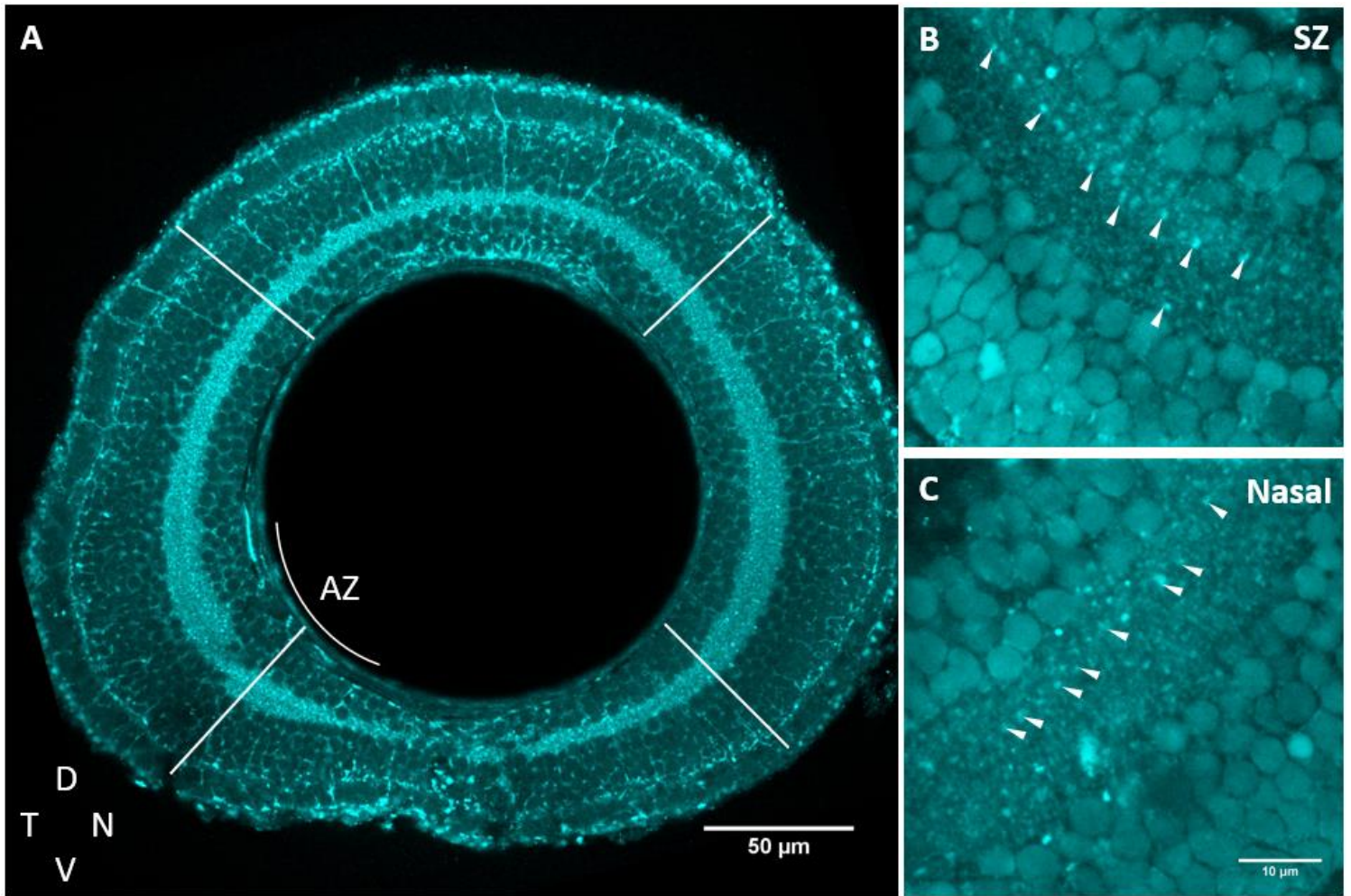


Figure 3: Region-specific distributions of mitochondria within the IPL.

(A) Sagittal section across 7 dpf larval retina, stained for Cox-iv, a mitochondrion-specific antigen. White lines roughly denote the regions considered D, N, V, T, and AZ for analysis. (B-C) Enlarged sections from (A) showcasing the different allocations of mitochondria within SZ (B) and nasal IPL (C). White arrows indicate punctae which would have been picked up by our automated method for quantification.

3. CHARACTERISING AN RGC PROMOTER SYSTEM

3.1 Historical background of the *Islet2b* promoter's discovery, uses, and applicability for studying RGCs

To relate cell structure and function in a genetically tractable organism like the zebrafish, one would ideally use a promoter which meets the following criteria: 1) it expresses universally among all cells in the target population; 2) it drives uniform expression among the various types within that population, and; 3) its expression is restricted exclusively to the cells of interest, at least in the areas in which they and their postsynaptic targets are located. For RGCs in the zebrafish retina, the promoter historically assumed to best match to this description is *Islet2b*, a LIM/homeobox transcription regulator expressed in the developing eye, tectum, trigeminal ganglia, posterior lateral line ganglia, and hindbrain (Tokumoto et al, 1995). However, although one of the most widely used promoters to study RGCs (Ben Fredj et al, 2010; Nikolaou et al, 2012; Robles et al, 2014), to date expression under *Islet2b* expression has not been systematically assessed, leaving open the possibility that it fails to meet one or more of the ideal qualifications.

For example, displaced amacrine cells, so-called because of the 'displaced' location of their somata within the GCL, have been described in many vertebrates, including the zebrafish (Hughes & Wieniawin-Narkiewicz, 1980; Perry & Walker, 1980; Wassle et al, 1987; Connoughton, V. & Dowling, J., 1998; Yazulla & Studholme, 2001; De Sevilla Muller et al, 2007; Kao & Sterling, 2006; Munoz et al, 2014). It is possible that *Islet2b* is expressed by one or more dAC types in addition to RGCs; and obviously, a promoter which does not selectively label RGCs would make for a less-than-ideal driver for studies employing it to characterize this cell population's suite of anatomical and functional properties, although not necessarily a disqualifying one. Of equal importance, it is unclear whether *Islet2b* is expressed by all RGCs, and if not, then what fraction of RGCs are *Islet2b*-positive.

In order to address these questions, we set out to test: first, the universality, uniformity, and specificity of *Islet2b* expression in RGCs using a combination of transgenic labeling, immunohistochemistry, and lipophilic dye tracing, and; second, its ability to drive functional reporters, e.g. for *in vivo* calcium imaging. Our results provide several key insights into the *Islet2b* expression profile. In particular, we validated the promoter's applicability for studying the

anatomy and function of RGCs in the larval retina, as well as characterizing several minor limitations of its use.

3.2 Materials and methods

Animal care and lines. All procedures, conditions, and non-transgenic zebrafish lines are as described previously (see chapter 2, section 2.2: “Animal care and lines”). In addition, a *Tg(Islet2b:nls-trpR, tUAS:MGCamp6f)* line was generated by injecting a mixture of Islet2b:nls-trpR and trpR:MGCamp6f tol2 plasmids with tol2 transposase RNA into one-, two-, and four-cell stage embryos. Embryos were screened for mCherry heart marker and GFP fluorescence in the brain after 48 *hpf* and raised to adulthood (F0), outcrossed with WT fish, and F1 larvae were selected based on strong GFP expression in the brain. Promoter and reporter plasmids were made using the Gateway system (ThermoFisher, 12538120) with combinations of entry and destination plasmids (AddGene) encoding the following sequences: p5E-Islet2b (Pittman et al., 2008), pME-nls-trpR (Suli et al., 2014), p3E-polyA (Kwan et al., 2007), and pDestTol2pA2 (Kwan et al., 2007) for the Islet2b:nls-trpR plasmid; p5E-tUAS (Suli et al., 2014), pME-MGCamp6f, p3E-polyA (Kwan et al., 2007), and pBH (Yoshimatsu et al., 2016) for the trpR:MGCamp6f plasmid. Plasmid pME-memGCaMP6f was generated by inserting a PCR-amplified membrane-targeting sequence from GAP-43 (Kay et al., 2004) into a pME plasmid and subsequently inserting a PCR-amplified GCaMP6f (Chen et al., 2013) at the 3' end of the membrane targeting sequence.

Tissue preparation, immunolabeling, and confocal imaging. For nuclear and membrane labeling, GFP immunostaining, confocal imaging, and image processing and analysis, all methods are the same as described previously (see chapter 2, section 2.2: “Tissue preparation, immunolabeling, and imaging”). GABA immunostaining was performed using rabbit anti-GABA (Sigma, A2052, 1:500) according to the protocol described by Jusuf and Harris (2009). Briefly, whole retinas were fixed in 2% paraformaldehyde/2% glutaraldehyde for 24 hours at 4°C, rinsed in PBS, treated with 0.1% sodium borohydride (NaBH₄) in 0.2% Triton X-100 in PBS for 10 minutes at room temperature, and rinsed again to remove excess NaBH₄. For immunolabeling, all steps are as described in chapter 2, with the following exceptions: blocking buffer consisted of 10% normal donkey serum, 0.1% Tween-20, and 0.5% Triton X-100 in PBS; primary and secondary antibodies were also diluted in this blocking buffer.

Thickness profiling. For determining the GCL and IPL thickness profiles, we took single-plane confocal images of *Tg(Islet2b:nls-trpR, tUAS:MGCamp6f)* retinas immunolabeled against GFP and stained with nuclear (Hoescht 33342) and membrane (Bodipy) markers. 2D width profiles were generated using custom-written scripts in IGOR Pro 6.37 (Wavemetrics), with Hoescht nuclear stain used to determine total GCL width, Bodipy membrane stain used to determine total IPL width, and GFP immunofluorescence used to capture the extent of *Islet2b* expression in the GCL and IPL. The thickness of the GCL as given by Hoescht was compared to the extent of the GCL GFP signal, while the IPL thickness given by Bodipy was similarly compared to the extent of the IPL GFP signal.

Axonal tracing. The lipophilic tracer dye DiD (Invitrogen, D307) was used to trace RGC axons from the retina to their arborization fields in the pretectum and tectum. 1 mg/mL stock solution was prepared in dimethylformamide and stored at -20°C. For injection into *Tg(Islet2b:nls-trpR, tUAS:MGCamp6f)* retinas, the lenses of whole fixed larvae removed and a sufficient amount of tracer dye injected into one of either the left or the right eye so as to completely cover the exposed surface of the GCL. Tissue was then incubated at 37°C for 3 days to allow the dye time to diffuse all the way up RGC axons to their terminals in the midbrain.

Two-photon imaging and stimulation parameters. For all *in vivo* imaging experiments, we used a MOM-type two-photon microscope (designed by W. Denk, MPI, Martinsried (Euler et al., 2013); purchased through Sutter Instruments/Science Projects) equipped with the following: a mode-locked Ti:Sapphire laser (Chameleon Vision-S, Coherent) tuned to 927 nm for imaging GFP and 960 nm for imaging mCherry/Bodipy in combination with GFP; two fluorescent detection channels for GFP (F48x573, AHF/Chroma) and mCherry/Bodipy (F39x628, AHF/Chroma), and; a water-immersion objective (W Plan-Apochromat 20x/1.0 DIC M27, Zeiss). For image acquisition, we used custom-written software (Scanm, by M. Mueller, MPI, Martinsried and T. Euler, CIN, Tuebingen) running under IGOR Pro 6.37 (Wavemetrics) taking image sequences at 64x48 pixel resolution (15.6 Hz, 2 ms line speed). For each functional scan, we first defined a curvature of the imaged IPL segment based on a structural scan, and thereafter “bent” the scan plane accordingly (“banana scan”). This ensured that the imaging laser spent a majority of time sampling from the curved IPL and INL, rather than adjacent dead-space. The banana-scan function was custom-written under Scanm.

For light stimulation, we focused a custom-built stimulator through the objective, fitted with band-pass-filtered light-emitting diodes (LEDs) ('red' 588 nm, B5B-434-TY, 13.5 cd, 8°; 'green' 477 nm, RLS-5B475-S, 3-4 cd, 15°, 20 mA; 'blue' 415 nm, VL415-5-15, 10-16 mW, 15°, 20 mA; 'ultraviolet' 365 nm, LED365-06Z, 5.5 mW, 4°, 20mA; Roithner, Germany). LEDs were filtered and combined using FF01-370/36, T450/pxr, ET420/40 m, T400LP, ET480/40x, H560LPXR (AHF/Chroma). The final spectra approximated the peak spectral sensitivity of zebrafish R-, G-, B-, and UV-opsins, respectively, while avoiding the microscope's two detection bands for GFP and mCherry/Bodipy. To prevent interference of the stimulation light with the optical recording, LEDs were synchronized with the scan retrace at 500 Hz (2 ms line scans) using a microcontroller and custom scripts. Further information on the stimulator, including all files and detailed build instructions can be found at <https://github.com/BadenLab/Tetra-Chromatic-Stimulator>.

Stimulator intensity was calibrated (in photons per second per cone) such that each LED would stimulate its respective zebrafish cone type with a number of photons adjusted to follow the relative power distribution of the four wavelength peaks of daytime light in the zebrafish natural habitat (Zimmerman et al, 2018; Nevala & Baden, 2019) to yield 'natural white': red, 100% (34×10^5 ph/s/cone); green, 50% (18×10^5 ph/s/cone); blue, 13% (4.7×10^5 ph/s/cone); ultraviolet, 6% (2.1×10^5 ph/s/cone). We did not attempt to compensate for cross-activation of other cones. Owing to 2-photon excitation of photopigments, an additional constant background illumination of $\sim 10^4$ R^* was played during recordings (Euler et al., 2009; Baden et al., 2013). For all experiments, larvae were kept at constant illumination for at least 2 seconds after the laser scanning started before light stimuli were presented.

No power calculations were carried out to determine minimum sample size. Since upwards of a hundred data points were gathered from each fish, and the data which passed quality criteria for use in analysis consisted of pooled ROIS from multiple fish, our sample sizes were sufficiently large to yield aimed power values of >0.99 (Serdar et al., 2021).

In vivo light stimulation. In preparation for 2-photon recordings of light-driven RGC responses, larvae were immobilized in 2% low melting point agarose (Fisher Scientific, cat: BP1360-100), placed ventral-side down on a glass coverslip, and submersed in fish water. They were then injected with α -bungarotoxin (1 nL of 2mg/mL; Tocris, Cat. 2133) into the ocular muscles behind

the right eye, to prevent movements of the eye and body which would interfere with image acquisition. During photostimulation and recording, larvae were kept at a constant 26°C.

The only test stimulus used for this set of experiments was a ‘light flash’, in which each of the four LEDs was blinked simultaneously on and off at 3 second intervals. For image acquisition and analysis of the calcium signals, we used custom-written software (Scanm, by M. Mueller, MPI, Martinsreid and T.Euler, CIN, Tuebingen) running under IGOR pro 6.37 (Wavemetrics). To prevent interference of the stimulation light with the optical recording, LEDs were synchronized to the scanner’s retrace (Euler et al., 2019). No statistical methods were used to predetermine sample size. Extracted waveforms were partitioned by k-means clustering.

3.3 Results

***Islet2b* expression profile.** To characterize the expression of *Islet2b* among inner retinal cells at 7 dpf, we performed immunocytochemistry against GFP and GABA in *Tg(Islet2b:nls-trpR, tUAS:MGCamp6f)* retinas, in which *Islet2b* is used to drive a membrane-targeted version of the calcium sensor GCamp6f by means of the tryptophan repressor system (Suli et al., 2014). Labeling revealed that *Islet2b* expression extends throughout the full thickness of both GCL and IPL (**Fig. 3a-b**) as determined by comparison against nuclear and membrane staining, respectively (**Supplementary Fig. 3a-g**). Notably, the IPL width profile as given by *Islet2b*-positive cell processes matches that of BC terminals, reaching peak thickness in the nasal and acute zone (**Fig. 3b**). That *Islet2b* is expressed by most, if not all, RGCs in the larval retina was confirmed by anatomical tracing, with *Islet2b*-positive RGC processes constituting the greater part of DiD-labeled axons terminating in the tectum and pretectal arborization fields (**Fig. 3c-e**). It is, however, interesting that several AFs, including AF4, 8, and 9, are unlabeled by *Islet2b* fluorescence. This suggests (Robles et al., 2014) either that: 1) a subset of RGC types, lacking promoter expression, may be systematically missed, or; 2) a subset of RGC types, with reduced promoter expression, may drive promoter expression at insufficient levels to adequately label postsynaptic targets in the brain. We suggest the latter possibility as being more likely, since, despite its apparent near universality among RGCs, *Islet2b* expression is patchy, with different cells and even entire regions of the retina more brightly labeled than others (**Fig. 3a**). This effect is more pronounced among the somata in the GCL than in the IPL’s dendrites, which exhibited the layered organization typical

of the dendritic stratification profile of retinal cell types, although some nonuniformity of expression was observable here too.

While RGCs constitute the bulk of *Islet2b*-positive cells at 7 *dpf*, expression is not restricted to ganglion cells but includes four additional populations. The first set of non-RGC cells is made up of GCL somata immunoreactive for GABA (**Fig. 3d-g**), a marker of zebrafish dACs (Connaughton et al., 1999; Jusuf & Harris, 2009), indicating that amacrine cells are among the cohort of *Islet2b*-expressing cells in the GCL. A second, smaller set of non-RGC cells is found in the INL and have cell bodies which closely hug the outer edge of the IPL. Because of the location of their somata, as well as the expression of GABA by a large proportion of them, these cells are likely to be INL ACs (**Fig. 3d**). The third set is comprised of photoreceptors (**Fig. 3a**). The fourth set of cells is located outside the retina, with their somata positioned anterior to the optic tectum, in the area of the pretectal AFs (**Fig. 3c**). Thus, in addition to ganglion cells, *Islet2b*-expressing somata and processes in the eye and brain is made up of a small but noteworthy fraction of non-RGCs.

Functional profile of *Islet2b*-expressing inner retinal cells. In order to determine *Islet2b*'s suitability for functional studies, we measured the light-driven calcium activity of inner retinal cells in response to a simple 'search flash' stimulus, in which all four LEDs were turned on and off at 3 second intervals. Our recordings confirmed that *Islet2b*-driven expression of MGCamp6 is sufficient to register calcium differentials in all *Islet2b*-positive cells in the GCL (**Fig. 3g-i**). However, individual cells displayed compartmentalization in terms of which cellular regions responded most reliably; in particular, while dendritic responses were robust and highly consistent in both ON and OFF IPL layers, only ~40% of somata exhibited detectable changes in GFP fluorescence (**Fig. 3g**). Additionally, in line with the nonuniformity of reporter expression across different retinal regions, the responsiveness of GCL cells varied somewhat from area to area, with cells positioned in nasal and temporal zones responding more reliably than those in either ventral or dorsal.

3.4 Discussion

In this chapter, we have shown that *Islet2b* is expressed throughout the GCL and IPL, and that it drives sufficient expression of calcium reporters to obtain strong signals in the IPL and a subset of

GCL somata. Although the scope of *Islet2b*'s applicability for studying the anatomy and function of RGCs is limited by a small population of *Islet2b*-expressing amacrine cells and by an inhomogeneous expression profile among GCL somata, some level of non-specificity and nonuniformity is a common feature of promoters intended for use in studying particular cell populations. The two other promoters frequently used in surveys of the ganglion cell population, *elavl3/HuC* and *Ath5*, express in a larger fraction of GCL and INL ACs (Tokumoto et al, 1995), while the extent of their expression among RGCs is uncertain. Despite its drawbacks, therefore, *Islet2b* is the only known promoter whose expression is almost exclusively restricted to RGCs.

Additionally, our characterization of the *Islet2b* expression profile confirmed the long-standing notion that the promoter is a marker for most, if not all, RGCs in the 7 *dpf* retina. It is possible that any RGCs missing from the *Islet2b*-positive population, as well as the promoter's irregular expression pattern across different retinal regions, are the result of epigenetic silencing, to which transcription activator systems are prone (Goll et al, 2009; Akitake et al, 2011). The tryptophan repressor system we used in this study is purported to be unsusceptible to gene silencing, since it does not contain the methylation-prone CpG sites (Suli et al, 2014). However, other mechanisms of epigenetic control besides methylation exist in the cell (Tycko, 2000; Delcuve et al., 2009; Zhang et al., 2019), and the *tUAS* sequence is unlikely to be entirely free from all silencing effects.

The reliability with which *Islet2b* drives functional expression in RGC dendrites is another highly serviceable feature of the promoter. Although the calcium transients generated in dendritic structures will not have reached the level of integration as those in somata and axons, they nevertheless represent the sum of input obtained from bipolar and amacrine cell processes, and thus the first level of RGC-specific signals. Additionally, that only a subset of somata exhibit observable responses, while precluding population studies in the GCL, makes our *Tg(Islet2b:nls-trpR, tUAS:MGCamp6f)* a candidate line for use in single-cell physiological studies, since individual somata are easily distinguishable from one another. It may also be possible to obtain universal functional expression among RGCs using *Islet2b* and a cytoplasmic- or nuclear-localized calcium sensor, since membrane-bound versions of GCamp will be unable to efficiently record calcium waves in cell body and axonal structures lacking calcium channels (Leterrier, 2018).

Therefore, we concluded that *Islet2b* is a suitable candidate for our study of RGCs. Having characterized the promoter's expression, we set out to perform the first full-scale survey of how

RGC structure (chapter 4) and function (chapter 5) varies in the AZ in comparison to other retinal regions.

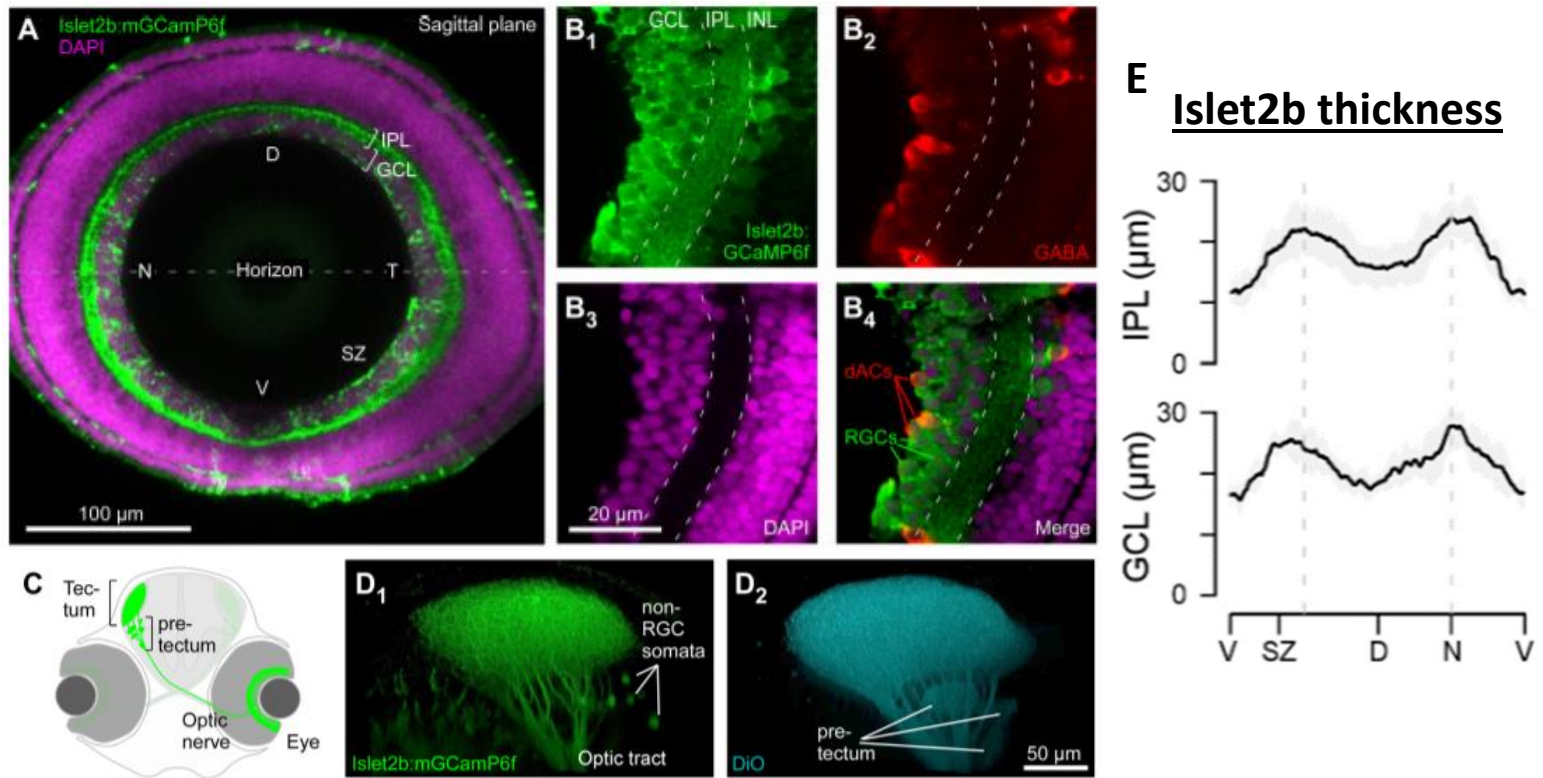


Figure 1: *Islet2b* expression profile in the larval zebrafish eye and brain.

(A) Sagittal section across 7 dpf *Tg(Islet2b:trpR-nls, tUAS:MGCAMP6f)* retina, stained for GFP (green) and Hoescht nuclear stain (magenta). T, temporal; SZ, acute zone; V, ventral; N, nasal; D, dorsal; INL, inner nuclear layer; GCL, ganglion cell layer. **(B₁₋₄)** Higher magnification images from SZ, including both *Islet2b* and nuclear stains as well as GABA (red) for GCL dACs and INL ACs. Note the subset of somata co-labeled for *Islet2b* and GABA **(B₄)**. **(C)** Schematic showing contralateral ganglion cell projections across the optic tectum to the brain, where they innervate the pre-tectum and tectum. **(D₁₋₂)** Confocal images of GFP signal **(D₁)** and DiD counterlabeling via injection into the eye **(D₂)** of *Tg(Islet2b:trpR-nls, tUAS:MGCAMP6f)* tectum/pre-tectum. Note that the pretectal areas, captured by the DiD stain, are not fully represented in the GFP channel. **(E)** *Islet2b* thickness profile in IPL and GCL, which doubles in thickness in the nasal and SZ relative to ventral and dorsal.

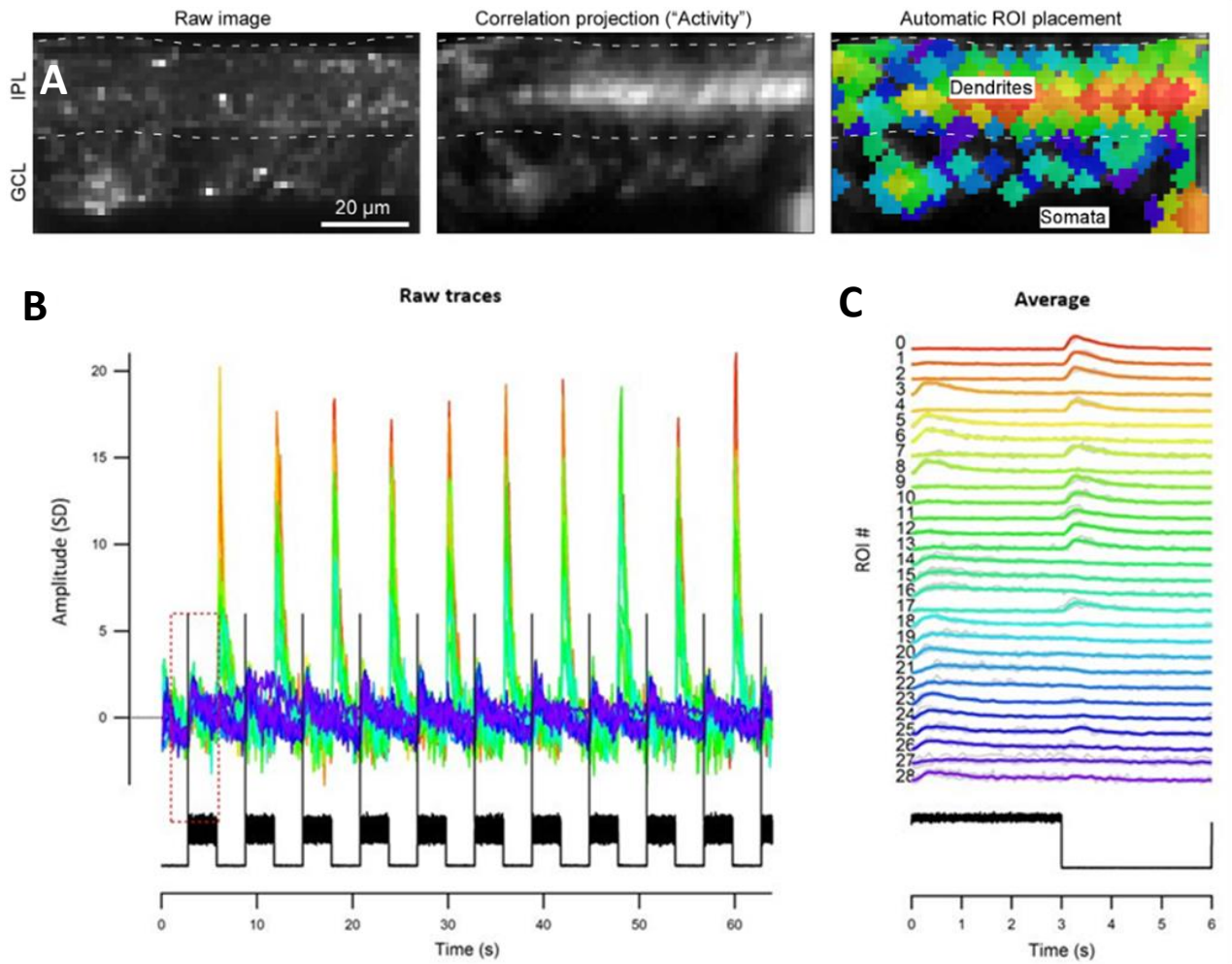


Figure 2: Functional profile of *Islet2b*-expressing cells.

(A) Sample scan showing automated ROI placement. The scan was manually segmented into IPL and GCL (white lines) and the mean correlation over time between all pairs of neighboring pixels computed; this correlation projection was then used to seed and demarcate ROIs by color according to activity level, from red (robust response) to purple (small response). (B-C) Sample traces extracted from IPL and GCL ROIs (B) and average of individual traces (C). Note that traces in (B-C) do not correspond to the ROI maps in (A).

4. MORPHOLOGICAL CHARACTERIZATION OF RGC TYPES

4.1 Introduction

Coincident with their role as the sole information highway between eye and brain, RGCs also function as the final stage in retinal visual processing (Wassle, 2004; Masland, 2012). Once visual information has been transmitted from PRs to INL interneurons, with the signals being shaped and decorrelated every step along the way, this welter of biological bits encoding brightness and contrast over wavelength, time, and space is received by ganglion cells for further feature extraction. Just as the types of PRs from which a BC receives its input define its polarity, contrast, temporal, and spectral tuning (Euler et al., 2014; Franke et al., 2017), RGC dendrites stratify selectively within the IPL laminae so as to sample BC axon terminals and transmit signals derived from specific combinations of presynaptic glutamate-release patterns (Masland et al., 2012; Euler et al., 2014). In concert with the intermediary influence of ACs, RGCs are tasked with further breaking down these signals into color, speed, and orientation components, extracting these stimulus characteristics by selectively responding, for example, only to a set of wavelength-specific channels when they also exhibit certain concomitant features such as size or speed. This information is then forwarded to integration centers in the brain in the form of multiple, parallel representations of each visual scene, with each representation corresponding to a distinct set of visual features (Masland, 2012).

In terms of how many such visual feature channels retinorecipient brain areas receive, the limiting factor is the number of distinct RGC types capable of conveying functionally distinguishable information (Baden et al., 2016). Although classification schemes for types has historically been a matter of contention (Rowe & Stone, 1977; Rodieck et al., 1983; Seung & Sumbul, 2014; Sanes & Masland, 2015), RGCs of a particular idealized type are assumed to share the same physiological properties, dendritic morphology, intra-retinal connectivity, retinal mosaic, and genetic and immunohistochemical markers (Roska & Werblin, 2001; Rockhill et al., 2002; Badea & Nathan, 2004; Kong et al., 2005; Volgyi et al., 2009; Sumbul et al., 2014; Kölsch et al., 2020). There is some evidence that axonal projection patterns are also type specific (Xiao & Baier, 2007; Robles et al., 2013; Morin & Studholme, 2014; Robles et al., 2014). Key here is the notion that cells displaying the same morphological characteristics and connectivity patterns within the visual circuit will have broadly similar response profiles, an hypothesis which has a creditable amount of

support from studies of individual cells in several vertebrate species (Cleland et al., 1975; Famigliesse & Kolb, 1976; Famigliesse et al., 1977; Roska & Meister, 2014). Nevertheless, it is a notion which has only begun to be tested with the rigor it merits following the advent, within the past decade, of techniques for large-scale genetic and functional profiling (Baden et al., 2016; Franke et al., 2017; Vishwanathan et al., 2017; Zimmerman et al., 2018; Yoshimatsu et al., 2019; 2020; 2021; Kölsch et al., 2020).

To date, the only large-scale attempts to classify RGS in the zebrafish retina have been in terms of gross anatomy. In 2002, Mangrum and colleagues proposed that the adult retina possesses 11 RGC types based on dendritic morphology and stratification on BC terminals within the IPL, although this number has been upped to 14 for larvae, based on single-cell fluorescence labeling (Robles et al., 2014). Robles and colleagues (2014) also identified 20 highly stereotypical RGC projection patterns which innervate the extraretinal AFs (AF1-9) and AF10/11 tectal sublaminae in a (morphological) type-specific manner, bringing the number of structurally-defined RGCs possessing unique combinations of dendritic morphology and axonal projection targets up to ~53. However, both studies were limited by the use of nonspecific labeling techniques¹, leaving open the possibility that these numbers do not represent the full morphological diversity of zebrafish RGC types. For example, many cells share a common dendritic structure (monosynaptic, bistratified, tristratified, and diffuse) but differ in their IPL depth, stratification profile within IPL laminae 1-6– which itself is not uniform between different regions, further complicating an already complex picture– and the number and type of BC/AC synapses. Any and all of these further possible RGC type-specific characteristics would likely have consonant functional implications.

Additionally, Mangrum and colleagues (2002) noted that the RGC types they described in their study appeared to lack the mosaic arrangement characteristic of most vertebrate species (Collin, 2008). Rather than the cells of given type having an approximately equidistant spacing with respect to one another, they appear to be irregularly arranged with no apparent ordering. But if, as Robles and colleagues (2014) claim, the combination of RGC type-specific inputs visual brain areas receive is determined in part by the location of their somata within the retinal hemisphere, then

¹ Mangrum and colleagues (2002) utilized the painstaking process of backfilling the lipophilic tracer dye DiI in individual axons in the optic nerve. On the other hand, Robles and colleagues (2014) made use of genetic mosaic labeling. Both research groups then had to screen for the subset of positive fish with sufficiently sparse labeling to permit reconstruction of individual, clearly defined cells.

presumably also the various functional properties associated with specific, differentially-innervated tectal regions (Hunter et al., 2013; Lowe et al., 2013; Nikolaou et al., 2013; Wang et al., 2019; Yin et al., 2019) are correlated with the functional response profile of particular retinal regions. Whether this effect is due to the differential distribution of RGC types, as the results of Mangrum and colleagues' study suggests, or as the result of cells of a given type activating differently depending on their location within the retina, is unknown.

To probe the anatomical diversity and distribution of RGC types, we performed single-cell photoactivation labeling under the RGC promoter *Islet2b* (see chapter 3). This method allowed us to map dendritic structures, location and number of synaptic densities (SDs), and soma position for individual, hand-picked RGCs, and to assess the cytoarchitecture characteristic of different parts of the eye. As the major part of this broad survey of ganglion cell morphology, we analyzed 131 RGCs from two regions, the nasal and AZ to quantitatively compare between cells responsible for conveying information about distinct parts of the visual world. Our findings reveal striking morphological diversity between nasal and AZ RGCs, with those surveying the backwards horizon featuring widefield and narrowly-stratified dendritic trees, while those positioned in the area temporalis were typically small-field and diffuse.

4.2 Materials and methods

Animal care and transgenic lines. All procedures, conditions, and non-transgenic lines are as described previously (see chapter 2, section 2.2: “Animal care and lines”). In addition, the transgenic lines *Tg(Islet2b:nls-trpR, tUAS:SyjRGeco1a)* and *Tg(tUAS:paGFP)* were generated by injecting the following plasmids into one-, two-, and four-cell stage embryos: for *Tg(Islet2b:nls-trpR, tUAS:SyjRGeco1a)*, a mixture of pTol2pA-islet2b-nlsTrpR (see chapter 3, section 2.2: “animal care and transgenic lines”; see also Janiak et al., 2019) and pTol2CG2-tUAS-SyjRGeco1a; and for *Tg(tUAS:paGFP)*, pTol2BH-tUAS-paGFP. Plasmids were constructed using the Gateway system (ThermoFisher, 12538120) with combinations of entry and destination plasmids (AddGene) encoding the following sequences: p5E-Islet2b (Pittman et al., 2008), pME-SyjRGeco1a, p3E-polyA (Kwan et al., 2007), and pDestTol2CG2 for the pTol2CG2-tUAS-SyjRGeco1a plasmid; p5E-tUAS (Suli et al., 2014), pME-paGFP, p3E-polyA (Kwan et al., 2007), and pDestTol2BH for the pTol2BH-tUAS-paGFP plasmid. pME-SyjRGeco1a was constructed by

inserting PCR amplified zebrafish synaptophysin without stop codon (Dreosti et al., 2009) followed by PCR amplified jRGeco1a fragment (Dana et al., 2016) into the pME plasmid. Similarly, pME-paGFP was constructed by inserting a PCR-amplified sequence encoding paGFP into a pME plasmid.

Tg(Islet2b:nls-trpR, tUAS:SyjRGec11a) embryos were screened for mCherry fluorescence in the brain and *Tg(tUAS:paGFP)* embryos for mCherry heart marker after 48 *hpf* and raised to adulthood (F0), outcrossed with WT fish, and F1 larvae were selected based on strong GFP expression in the brain. Expression of paGFP was then obtained by crossing these two lines. Using this combination, RGCs co-express SyjRGeco1a with paGFP, but the red calcium indicator did not interfere with green channel for paGFP.

Experimental setup, photoactivation, and two-photon imaging. The 2-photon microscope used for *in vivo* photoactivation experiments has been described previously (See chapter 3, section 3.2: “Two-photon imaging and stimulation parameters”). Prior to photoactivation, larvae were immobilized in 2% low melting point agarose (Fisher Scientific, cat: BP1360-100), placed ventral-side down on a glass coverslip, and submersed in fish water. They were then injected with the following solutions: 1) α -bungarotoxin (1nL of 2mg/mL; Tocris, Cat. 2133) into the ocular muscles behind the right eye, to prevent movements of the eye and body which would interfere with image acquisition; 2) bodipy membrane dye (1nL of 1mg/mL; Sigma, D3821) into the space behind the right eye and underlying skin, to use as an anatomical landmark highlighting membranous areas of the GCL and IPL. Larvae were reoriented, placed right-side down so as to expose a sagittal view of the left eye, and subsequently left for 10-20 minutes at 25°C to allow the dye to diffuse into the retina. After approximately 20 minutes, the IPL was uniformly stained, while the stain in the GCL had a swiss cheese character, with dye in the outer membrane outlining circular unstained patches corresponding to RGC and dAC nuclei.

Using the holes within the GCL as a guide to where the cell bodies were located, we selected for photoactivation 2-5 cells per eye in one of four regions (Fig. 4a): most activated cells were in either nasal or acute zone, with a smaller fraction in dorsal and ventral for morphological comparison. Only somata were chosen which were a minimum distance of 30 μ m from previously-photoactivated cells. For photoactivation, a 760 nm laser was focused on a single somata at a time for a period of ~2 minutes, after which the larva was left >40 minutes to allow the activated GFP

adequate time to diffuse up into the dendrites. For visualization and imaging, the laser was brought back up to 927 nm, and stack 512x512 pixel images taken which encompassed each cell's soma, axon initial segment, and the entirety of the dendritic structure. All images were taken at the same magnification to allow morphological comparison between cells. Throughout, the Bodipy signal was included as an anatomical reference.

Data analysis. General image processing and type quantifications were performed in IGOR pro 6.37 (Wavemetrics), Fiji (NIH), and Excel (Microsoft), respectively.

Digitizing photoactivated cells for quantitative comparison of nasal and AZ RGCs. For mapping dendritic swellings (taken as a proxy for SDs) in photoconverted GCL cells, image stacks from the GFP channel were converted to 8-bit binary and the background fluorescence subtracted and smoothed; for background subtraction, the same rolling-ball radius was used for all images. Stacks were then thresholded automatically, and any remaining neurites that clearly did not belong to the most strongly labeled cells were manually removed so that only the soma and SDs of each target cell remained. To map somata and SDs in three-dimensional space, the 3D Objects Counter plugin in Fiji was used. The 3D positions of all detected objects, represented as center-of-mass coordinates, were then normalized relative to the boundaries of the IPL as determined by the Bodipy counterstain in the mCherry channel. This generated an IPL-aligned 'dot-cloud' for each RGC, with each cell's soma and SDs located with respect to their position below and within the IPL, respectively. This dot-cloud was then used as the input for a custom clustering algorithm. We also projected each dot-cloud into *en-face* and sideview density maps for visualization. Note that sideview projections shown in **Fig. 2A** (rightmost) and **Fig. 2B** are laterally compressed five-fold to highlight differences in stratification depths across the IPL.

Dot cloud-based clustering of nasal and AZ RGCs. A custom MatLab script was used to extract basic metrics about morphology from the dot-clouds generated from 131 RGCs (n=67 from nasal and n=64 from AZ). For all analysis, the coordinate axes are orientated such that the y-axis is perpendicular to the plane of the retina, spanning the width of the IPL, while the x- and z-axes are tangential to the plane of the retina, with the x-axis representing cross-sectional length along the IPL band and the z-axis the depth in the sagittal plane from lateral to medial. The coordinates in the y-dimension are scaled so as to lie in the interval [0,10] for any processes within the IPL, while >10 was used to designate processes extending into the INL and <0 for processes within the GCL,

where applicable. The position of the soma, which always lay in the GCL, was not used for clustering.

Three summary statistics, each of which capture some aspect of the dendritic architecture, were defined for use in clustering: 1) `y_span`: the width of the dendritic tree in the y-direction; 2) `y_mean`: the mean position of the points in the dendritic tree in the y-direction, and; 3) `num_pts`: the number of points in the dendritic tree. Prior to settling on these as the final summary statistics to be used to represent our data and in extracting statistics, we tried several other summary statistics; however, the three summary statistics defined above proved sufficient to capture RGC dendritic structures with enough accuracy to differentiate their point clouds into the basic RGC morphological groups. We nevertheless did define one further summary statistic, the `xz_area`, representing the area spanned by the dendritic tree in the xz-plane, calculated as the convex hull using the Matlab routine **`convhull`**. While this statistic was not used for clustering, since the information conveyed by the xz-area statistic is largely captured between `y_span` and `num_pts`, it is important in that it captures important characteristics of the dendritic morphology. These aspects, represented by this fourth summary statistic, are depicted in the results section alongside `y_span`, `y_mean` and `num_pts`.

Prior to clustering, each of the summary statistics was standardized by subtracting the mean and dividing by the standard deviation. In this way, we ensured that each of the summary statistics was equally weighted by the clustering algorithm. Clustering was then performed in two stages, using an agglomerative hierarchical method in both cases. This hierarchical clustering was performed using the Matlab routines **`pdist`**, **`linkage`** and **`cluster`**, which perform the following functions: the function **`pdist`** calculates the distances between each RGC in (`y_span`,`y_mean`,`num_pts`)-space; the function **`linkage`** then takes the output of the **`pdist`** routine in order to produce an agglomerative hierarchical cluster tree. We selected to use the ‘city block’ distance metric for **`pdist`** and the ‘average’ distance metric for **`linkage`**, since these resulted in a larger cophenetic correlation coefficient (CCC) than any other combination of distance metrics. The CCC is a measure of the fidelity with which the cluster tree represents the dissimilarities between observations. It was calculated using the Matlab routine **`cophenet`** and takes values between [-1,1], where values closer to positive unity represent a more faithful clustering. In the results presented here, the first stage of clustering had a CCC of 0.77 (2 d.p.), while the two subclusterings in the second stage had

CCCs of 0.77 (2 d.p.) and 0.83 (2 d.p.). Lastly, RGCs were assigned to clusters using the Matlab routine **cluster**. The number of clusters was determined by specifying a cutoff distance which was chosen following visual inspection of the cluster tree dendrogram so as to respect a natural division in the data.

The first stage of clustering used all three summary statistics (y_{span} , y_{mean} and num_pts), splitting the data into 18 clusters. Two of the resulting clusters were large, and a cursory examination showed that they contained a variety of morphologies. The subsequent round of hierarchical clustering used the y_{span} summary statistic alone to split the 18 initial clusters, the first cluster into 6 subclusters and the second into 3 subclusters, for a total of 25 clusters. Of these, the 13 clusters containing a minimum of 4 members were included in our final dataset.

Dot cloud-based quantification of dendritic tilt. To quantitatively demonstrate dendritic polarization, we calculated the center of mass of each SD point cloud as the mean of each point cloud's cumulative x , y and z positions. We then transformed these values from Cartesian to spherical polar coordinates with the origin centered at the soma (given as r, θ, φ : where $r > 0$ (μm) is the distance of the dendritic center of mass from the soma; the polar angle, $0 \leq \theta \leq \pi$ (rad), represents the dendritic tilt strength such that $\theta = 0$ corresponds to no polarization relative to the somatal origin and $\theta = \pi/2$ corresponds to the the dendritic center of mass's having the same IPL or GCL depth as the soma; the azimuthal angle, $0 \leq \varphi < 2\pi$ (rad), represents direction of the dendritic tilt. Note that the relationship between our Cartesian and spherical polar coordinate systems is different from standard representations in that we have swapped the y and z axes, such that the polar angle is subtended from the y -axis, rather than from the z -axis.

We used the two-sample Kolmogorov-Smirnov test to check whether the positions of nasal and AZ SD point cloud center of masses relative to somata in each of the r , θ and φ dimensions are from the same continuous, unidimensional probability distribution. For this, we used the Matlab routine **kstest2** for r and θ , and the **circ_kuipertest** routine from the CircStat toolbox (Berens, 2009) for φ , since this variable is (2π) -periodic. In comparing AZ and nasal RGCs, the test's predications for each of the three variables are as follows: the dendritic center of mass positions, r , are predicted to be from different distributions ($p = 0.0209$, 3 significant figures); the dendritic tilt strengths, θ , are predicated to be from the same distribution ($p = 0.894$, 3 significant figures); and the dendritic tilt angles, φ , are predicted to be from different distributions ($p = 0.001$).

4.3 Results

RGC cytoarchitecture varies systematically across the retina. To assess the dendritic cytoarchitecture of individual RGCs across the retina, we utilized a photoactivatable GFP (paGFP: Patterson & Lippincott-Schwartz, 2002) expressed under *Islet2b* (see chapter 3). Individual ganglion cell somata were photoconverted at random in four non-overlapping regions of the retina: nasal (~45%), acute zone (~45%), dorsal (~7%), and ventral (~3%) (**Fig. 1A-B**). A total of n=243 cells from n=113 fish were converted and imaged. After discarding n=3 dACs which had no distinguishable axon, and another n=93 RGCs which overlapped with neighboring photoconverted cells or for which the tissue died before photoactivation was complete, a final total of n=147 RGCs from the four retinal regions were retained for further analysis.

In addition to identifying most of the dendritic morphologies which Robles and colleagues (2014) reported (**Fig. 2B-C**), we found several other structurally distinct RGC types. These included cells with the characteristic mono-, bi-, and tristratified, and diffuse arborization patterns (Robles et al., 2013, 2014), but which stratified in or across different IPL layers than have been previously described, as well as cell types displaying more novel variants of the basic structures (**Fig. 3D**). Of this latter group, one type displayed a ‘basket’ morphology, with processes stratifying diffusely across the entirety of the IPL and studded with large, regularly interspersed punctae.

The distribution of each type showed a high degree of variance between the four regions surveyed. Those positioned in ventral and dorsal areas were almost exclusively monostратified or narrowly diffuse, with dendrites stratifying in only one or two IPL sublaminae, in consonance with the narrowing of the IPL as it approaches its ventral and dorsal extremes. By contrast, RGCs in the nasal and acute zone exhibited all four major morphological structures, although the relative proportions of each varied between the two regions. The acute zone contained a higher percentage of tristratified and diffuse morphologies (11% and 64% respectively) than did nasal (5% and 35% respectively), while the converse was true for mono- and bistratified morphologies, which were overrepresented in the nasal (44% and 16%, respectively) compared to the AZ (11% and 14%, respectively).

Additionally, cells of a given anatomical type displayed region-dependent architectural specializations. Most notably, the dendritic trees of virtually all nasal and acute zone RGCs had a distinctive ventral-to-dorsal orientation in the sagittal plane. That is, cells in both regions oriented their processes toward the dorsal part of the eye, only rarely stratifying ventrally or, in a few cases, lacking neuritic orientation altogether. The exceptions were primarily comprised of cells with diffuse dendritic trees, including the type we have identified as basket cells. By contrast, cells positioned in ventral and dorsal regions were much less asymmetric, with orientation preference decreasing progressively for cells positioned closer to either extremum. No dendritic asymmetry was observed for cells positioned approximately at either ventral or dorsal peak.

RGC synaptic density profiles display region-specific structural specializations. These striking biases in type distribution and dendritic morphology prompted us to ask whether other cytoarchitectural features of larval RGCs might also exhibit region-dependent specializations. For example, differences in the number, distribution, shapes, and sizes of postsynaptic densities (SDs) would affect intra-retinal connectivity between RGCs and their presynaptic partners, determine the physiology of individual synaptic contacts, and shape the response which the ganglion cell transmits to the brain. Mapping the locations of each cell's dendritic active sites would thus provide insight into the prospective functional capacity of RGCs in different retinal regions, as well as clarifying whether the anatomical variations discussed in the preceding section have functional implications.

To explore this possibility, we used the $n=64$ AZ RGCs and $n=67$ nasal RGCs which comprised the bulk of our paGFP dataset. First, we semi-automatically detected each RGC's soma and SDs, reconstructing each cell as a 'point cloud' in 3-dimensional space relative to its position in or below the IPL as determined by Bodipy counterstaining (**Fig. 2A**; Robles et al., 2014; Franke et al., 2017). We assumed that globular punctae, dendritic swellings of relatively higher mean fluorescent intensity than the rest of the RGC processes with which they were associated, corresponded to the cell's complement of SDs, since: 1) RGC dendrites lack spines (Wong et al, 1992; Sanes & Masland, 2015), such that ribbon synapses and conventional dendritic synapses serve as the sole areas of input from bipolar and amacrine cells, respectively (Dowling & Boycott, 1966; Dubin, 1970; Kolb, 1979; Raviola & Raviola, 1982; Nelson & Kolb, 1983; Sterling, 1983), and; 2) each puncta may represent one or multiple SDs, but only a single site of contact for a bipolar or amacrine

cell terminal. Although the link between dendritic swellings and SDs remains tenuous without further genetic and immohistochemical verification, this approach nevertheless provided a useful means of approximating the overall stratification profiles of RGCs in a quantitative and unbiased manner. In this manner, we were able to use the resultant SD point clouds to compare depth and density profiles of RGCs positioned between the two zones, and to extract basic metrics about their morphology, including dendritic tilt (**Fig. 2B-E**), stratification width (narrow or diffuse) (**Fig. 2F-H**), en-face dendritic area (**Fig. 2I**), and the total number of SDs (**Fig. 2J**).

Our model reliably preserved the stratification profiles of nasal and AZ RGCs while revealing key distinctions in their respective postsynaptic architectures (**Fig. 2**). In agreement with our morphological characterization, the SD profiles of cells in either region displayed a distinct ventral-to-dorsal tilt among linearly- and diffusely-stratifying arbors, resulting in retinotopically opposed orientations between the dendritic structures of nasal and AZ RGCs (**Fig. 9B-E**). Similarly, the number of RGCs whose SDs were dispersed diffusely throughout the IPL was higher in the acute zone than in nasal areas, with SD point clouds from ventrotemporal RGCs tending to be more widely distributed across the plexiform layer than those in the nasal (**Fig. 9F-H**). This effect was produced by a bias in AZ RGC point cloud structure, with SDs predominantly inhabiting lower areas of the IPL in preference to laminae fronting the inner edge of the INL. That is, among the cells surveyed, ON-stratifying, but not OFF-stratifying, AZ RGCs were more likely to be found diffusely stratifying across the IPL depth than were nasal RGCs. There was no apparent difference between the distribution of RGCs' en-face dendritic area (**Fig. 2I**) or the number of dendritic swellings possessed by each cell (**Fig. 2J**) between the two regions, suggesting that RGCs employ a set amount of cellular building material and rearrange it to suit their structural and functional requirements in a region-dependent manner (But see **chapter 2** for discussion of intercellular reapportioning).

Having confirmed the overall architectural patterns initially observed in the RGC dendritic structures, we next asked to what extent the SD point cloud stratification differences between AZ and nasal RGCs (**Fig. 2**) accorded with the presence of distinct morphological types between the nasal and AZ (**Fig. 3**). For this, we jointly clustered RGCs from both regions, taking into account their mean IPL depth, IPL depth distribution widths, and number of SDs. This yielded a total of 25 morphological clusters of which 13 with a minimum of $n = 4$ individual members were

considered for further analysis (**Fig. 3A**). In line with the current annotated, though position-independent, census for larval RGCs (Robles et al., 2014), as well as our own observations with regard to cells in different parts of the retina, the RGC clusters for the two regions exhibited diverse dendritic profiles including a variety of both narrowly (C_{1-7}) and diffusely stratified profiles (C_{8-13} , **Fig. 3B**). However, several clusters were mostly made up of RGCs coming from only one of the two retinal regions (**Fig. 3A**). For example, narrowly stratified clusters $C_{2,6,7}$ were dominated by nasal RGCs, while diffusely stratified clusters $C_{8-10,13}$ mainly comprised AZ-RGCs. In fact, the only narrowly stratified clusters that were dominated by AZ-RGCs were Off-stratifying $C_{1,3}$. Clusters $C_{3,5,10,13}$, which had the smallest dendritic areas, were comprised largely or entirely of temporally positioned ganglion cells, with the exception of C_5 which was equally represented by cells in either region (**Fig. 3D**). Thus, SD point cloud-mapping preserves the diversity of dendritic architecture found in different retinal locales, lending credence to the notion that a cell's structural features should be an indicator of their physiology.

4.4 Discussion

It has been proposed that attempts to categorize RGCs with primary reference to gross dendritic morphology have failed to capture their cytoarchitectural diversity, let alone the full set of distinct ganglion cell types (Morin & Studholme, 2014; Robles et al., 2014; Baden et al., 2016). The results presented in this chapter are in strong agreement with this notion. We have shown that RGCs in the larval zebrafish represent an anatomically diverse population exhibiting region-specific phenotypes, which appear most pronounced at the horizon.

Potential functional consequences of populating the AZ with small-field, diffuse RGCs. The most prominent structural feature differing across retinal regions is the dendritic tree itself, which we have linked to its synaptic density profile. Since they show a high degree of correspondence, the one can be used to predict the other, allowing tentative conclusions to be drawn about an RGC's physiology from its morphology, IPL stratification, and retinal position. For example, the relative abundance of diffuse dendritic arborizations among AZ RGCs suggests that they receive and integrate input from a large number of BC types distributed across multiple IPL layers, which may represent either receptivity to a wide range of BC response profiles, or the nuanced temporal and spatial precision offered by a system wherein BCs present numerous fine-scale variations within a

small signal range. Of primary interest here is the manner in which diffuse AZ RGCs tended to stratify their dendrites— and by extension the spread of their SDs— within the ON layers of the IPL, while by contrast those AZ RGCs with narrow stratifications (mono- or bistratified) were restricted to OFF laminae. This is strongly reminiscent of the upwards shift of the functional ON/OFF boundary and the concomitant compression of OFF IPL circuits among AZ BC terminals (Zimmerman et al., 2018). Thus, while the IPL thickness expands relative to the neighboring dorsal and ventral parts of the plexiform layer, it does so by preferentially expanding or multiplying ON laminae. This shift to ON at the expense of OFF circuits appears to be a general component in the ventrotemporal prey-detection pathway from PRs to forebrain (Semmelhack et al., 2014; Zimmerman et al., 2018; Yoshimatsu et al., 2019).

The small dendritic fields we find overrepresented among AZ RGCs in comparison to nasal RGCs is a fact which also has interesting functional implications. Typically, small-field dendritic structures in the retina are associated with correspondingly small spatial receptive fields which are activated by small dot-stimuli of either positive or negative contrast with respect to surrounding illumination, and a receptive field surround whose activation results in attenuation or full suppression of the cell's activity (Levick, 1967; Zhang et al., 2012; Jacoby & Schwartz, 2017). This is, broadly, the functional profile one would expect for a subset of cells geared toward detecting dot-like objects the same size or smaller than the receptive fields of cells of those types involved in this process; a description which immediately calls to mind the tiny, UV-bright microorganisms zebrafish larvae prey upon. The most obvious line of evidence in support of these RGCs playing such a role, and one which we have previously discussed (see **chapter 1, sections 1.4 and 1.5**), is that UV PRs positioned in the ventrotemporal retina respond preferentially to just such stimuli (Yoshimatsu et al., 2019). Additionally, the small-field, diffusely-stratified RGCs we have identified resemble one of the morphologically-defined RGC types whose axons innervate the pretectal area AF7/PSp, the main forebrain region activation of which is associated with prey capture activity (Semmelhack et al., 2014). Of course, since AF7 receives the greater part of its innervation from the temporal retina (Robles et al., 2014), it is unlikely that a mere one or two RGC types are alone responsible for the entirety of its axonal input from the retina. Indeed, in light of the results we have shown in this chapter, it is quite conceivable that many, or even most, of the diffuse RGCs within the AZ provide the activating influence on AF7 needed to induce prey capture

responses (Semmelhack et al., 2014), since the vast majority are, in terms of anatomy, classifiable as ON RGCs.

A final point of interest with regard to RGCs in general, and those positioned within the AZ in particular, is the relative abundance of morphologically distinguishable types. Out of the ~5000 cells comprising the larval zebrafish's ganglion cell population in each eye, only about 400 of these constitute the density hotspot we have been using throughout this thesis to delineate the AZ from the surrounding ventrotemporal environs. If it is indeed the case that well upwards of a dozen morphological RGC types are located within the AZ, this suggests that very low numbers of individual RGCs belonging to a given type—on the order of just 20 to 30 cells—are responsible for conveying prey-related information to midbrain visual regions. On a conceptual level, this is in agreement with other findings as regard processing of prey-related visual information, including the ability of single pretectal neurons to evoke prey capture behavior (Antinucci et al., 2019), as well as the finding that a single pretectal arborization field, AF7, appears to be responsible for mediating the transformation from sensory input to motor output in response to prey-like stimuli (Semmelhack et al., 2015).

Implications of the larval RGCs' asymmetric dendritic orientations. Although not an effect exclusively represented among AZ RGCs, it is of note that the greater part of the RGCs imaged for this study exhibited asymmetry with respect to the orientation of their dendritic structures, and that this polarity was position dependent. It is possible, of course, that this characteristic is a vestigial holdover of developmental processes, set up, for example, by proximity to the optic fissure. Yet, RGCs develop and move into place first of all retinal cells (Schmitt & Dowling, 1994, 1999), and none of the other cell type have this extraordinary degree of dendritic polarity at 7 *dpf*. Moreover, a fully developmental explanation may not sufficiently explain the extent of the specificity with which RGC dendritic tilts in different retinal regions varies. Research in other model organisms may provide a clue.

This asymmetry along the retinal axis is reminiscent to that of mouse JamB-positive (Kim et al., 2008; Joesch & Meister, 2016) or 'miniJ-type' RGCs (Rousso et al., 2016), comprising several direction-selective RGC types which, in addition to their dorsoventral tilt, stratify differentially between ON and OFF IPL layers (Sanes & Masland, 2015). Before rushing to draw conclusions from the parallels, it will be important to preface any such discussion by take noting of the

differences between the two species' respective RGC populations. First, in most cases the proximal dendrites of the zebrafish larvae's RGCs were perpendicular to the IPL rather than exiting the soma at a slant as do the mouse's JamB-RGCs. Second, JamB-RGCs make up only a subset of the mouse ganglion cell population, while in zebrafish larvae this dendritic asymmetry is almost universal among nasal and temporal RGCs, and to a lesser extent among those in ventral and dorsal as well. Finally, JamB-RGCs are oriented dorsal-to-ventral, while those in the zebrafish larva tend to orient in a ventral-to-dorsal direction.

Nevertheless, the similarities between these two species' RGCs are striking. Since, as we have shown, the neuritic stratification profile reflects the locations of an RGC's SD complement, it too would be expected to have consequences bearing on the cell's function, in terms both of the collective BC/AC input it receives and the cumulative signal it transmits to the brain. The most obvious possibility is that cells in the nasal and temporal zones, like JamB-RGCs, are direction and/or orientation selective in the up-down direction, and that this effect falls off moving to dorsal and ventral extrema. Zebrafish larva, when exploring, eating, and avoiding predators in their native Indian waters, are presented with a backdrop which moves to one side or the other more than it does up or down. One can begin to see, therefore, why they might require motion-selective cells responding preferentially to movement in the up-down direction, since this is more likely to be of behavioral relevance than the constant back-and-forth along the retinal horizon. The possibility that larval RGCs increase in motion-responsiveness toward the nasal and AZ, and what the ecological and behavioral implications of this might be, will remain for future studies to explore.

The next step: bridging the gap from form to function. These are tantalizing hints at what the functional profile of RGCs might be, and segway what up until this point has been for us largely a discussion of anatomy into the larger question of how neuronal architecture in the retina relates to its cells' activity in response to light stimuli of varying spatial, spectral, and temporal composition. With regard to the AZ, what is the range of responses of which its relatively small complement of RGCs is capable of responding to, and how closely does this activation profile match that of preceding retinal layers or of RGCs positioned in different parts of the inner retina? And how, in the final accounting, do these variations in function between AZ and non-AZ areas reflect the structural differences we have described in this chapter? In chapter 5, we turn to

functional imaging to address these questions, and discuss the implications of our results with regard to the larval zebrafish's ability to perceive and process prey-related information.

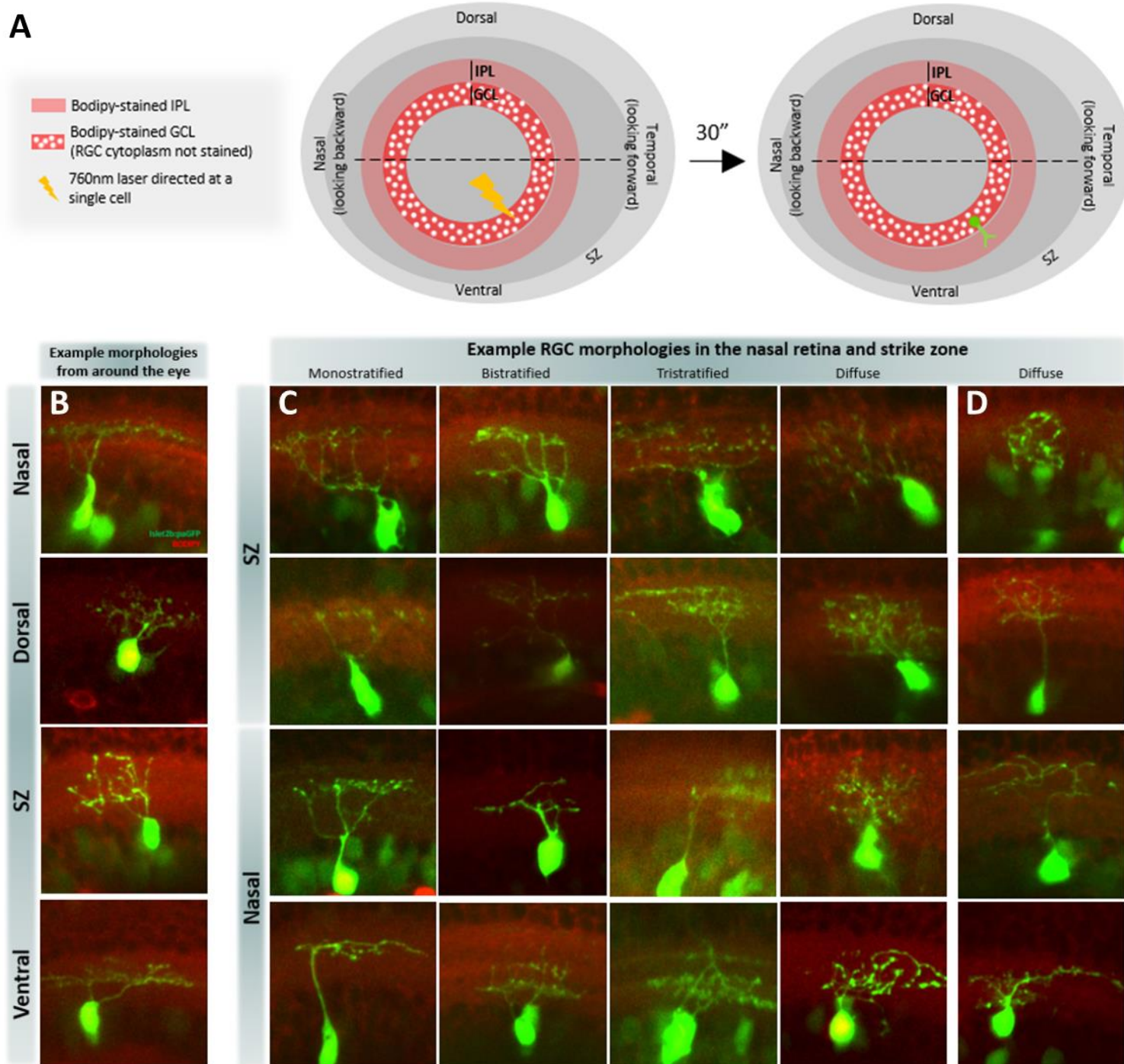


Figure 1: Photoactivation of single RGCs reveals cytoarchitectural variation across the retina.

(A) The photoactivation process uses a 760 nm-wavelength laser focused on a single soma at a time to photoconvert cells. GFP then diffuses throughout the cytoplasmic space of axons and dendrites over the course of 30-60 minutes. (B-D) Photoconverted cells in different eye positions differ in the relative proportions of dendritic architectural types as well as their degree of dendritic asymmetry.

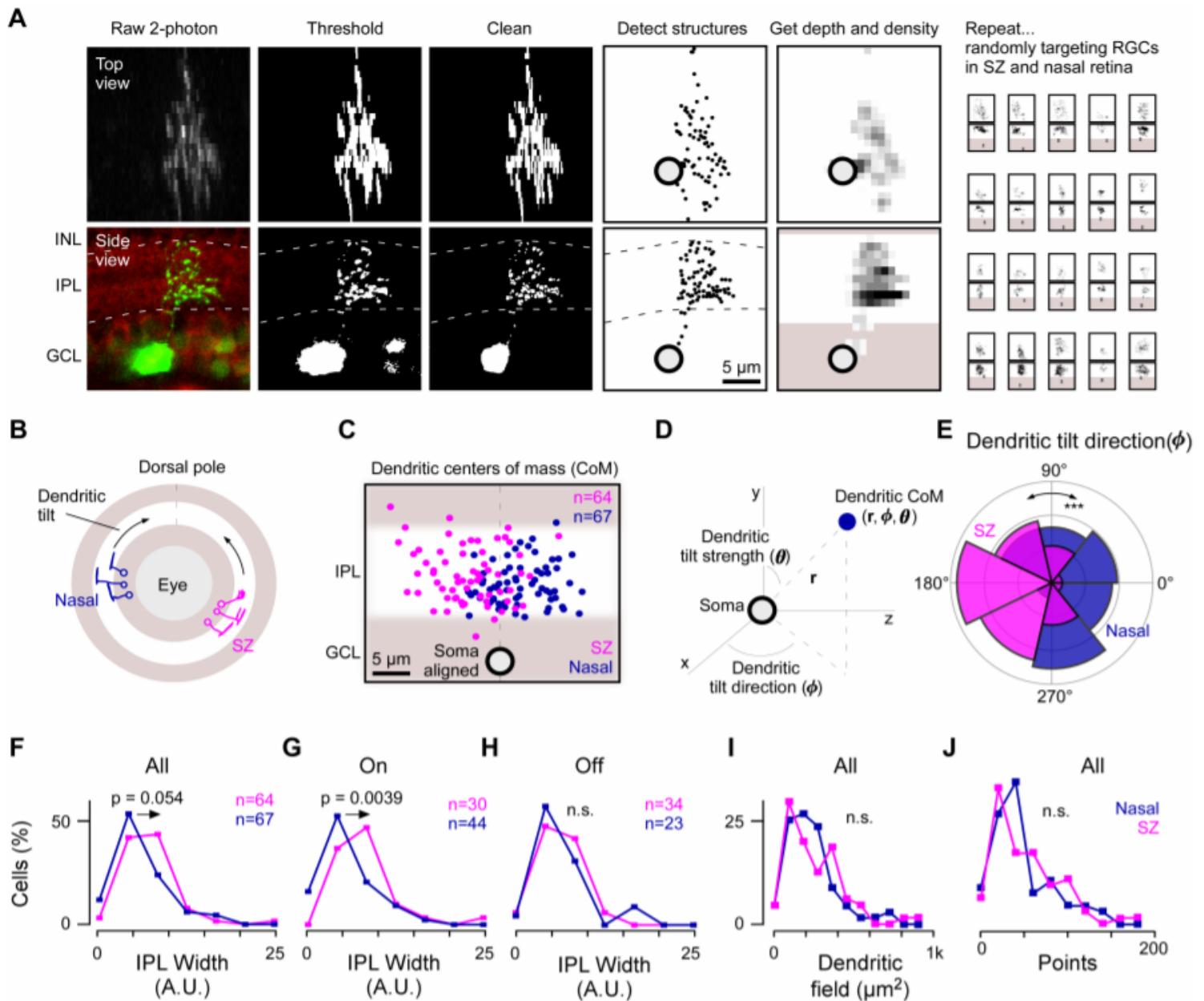


Figure 1: nasal and SZ RGC SD profiles are distinct.

(A) Workflow for mapping the locations of putative RGC SDs. Following photoconversion, stack GFP images of photoconverted cells (green) were aligned with Bodipy counterstain to demarcate the IPL boundaries (red), thresholded automatically, cleaned manually where required, and the positions of soma and SDs used to generate RGC point clouds. Finally, the point clouds from all nasal and SZ cells were used to quantify summary statistics, as well as to generate more easily-visualizable density maps showing the major axes of SD distribution. (B-J) Summary statistics and accompanying diagrams for nasal (blue) and SZ (magenta) RGC dot clouds. (B) Schematic of nasal and SZ RGC dendritic orientation. (C) Centers of mass for all nasal and SZ RGC dendritic arbors, with the value for each cell normalized to the location of its soma. (D) Representation of soma-oriented polar coordinate system used to quantify dendritic tilt for an individual RGC. This quantification used the Cartesian coordinates generated from each RGC's point cloud. r : distance in micrometers between a given cell's soma and dendritic center of mass. θ : strength of the dendritic tilt, ranging from 0° (no tilt) to 90° (maximal positive tilt). ϕ : direction of the dendritic tilt in retinotopic space, and viewed within the sagittal plane, from 0° to 360° (see figure legend for (E)). (E) Graphical demonstration of ϕ . To understand this, imagine dendritic polarization increasing from dorsal (where RGCs, having little or no tilt, will have a ~90° tilt) and ventral (similar to dorsal, differentiated here by being given a ~270° tilt) extrema as they approach either horizon, where 0° and 180° indicate opposing orientations. Therefore, for the right eye, a relatively higher number of nasal cells have a ~360° tilt, indicating a strong clockwise dendritic orientation, while by comparison a relatively high number of SZ cells have a 180° tilt, indicating a strong counterclockwise dendritic orientation. Note, however, that even RGCs falling around 90° and 270° had slight eccentricities, which is why pie chart slices are not oriented precisely at either pole. Size of slices represent relative proportions of cells. ϕ differed significantly between nasal and SZ RGCs. (F-J) Summary statistics for SD distributions for all RGCs (F), SD distributions for RGCs preferentially stratifying within the ON IPL sublaminae (G), SD distributions for RGCs those stratifying preferentially within the OFF IPL sublaminae (H), total en-face area of the SD distribution for all RGCs (I), and total number of SDs per RGC (J).

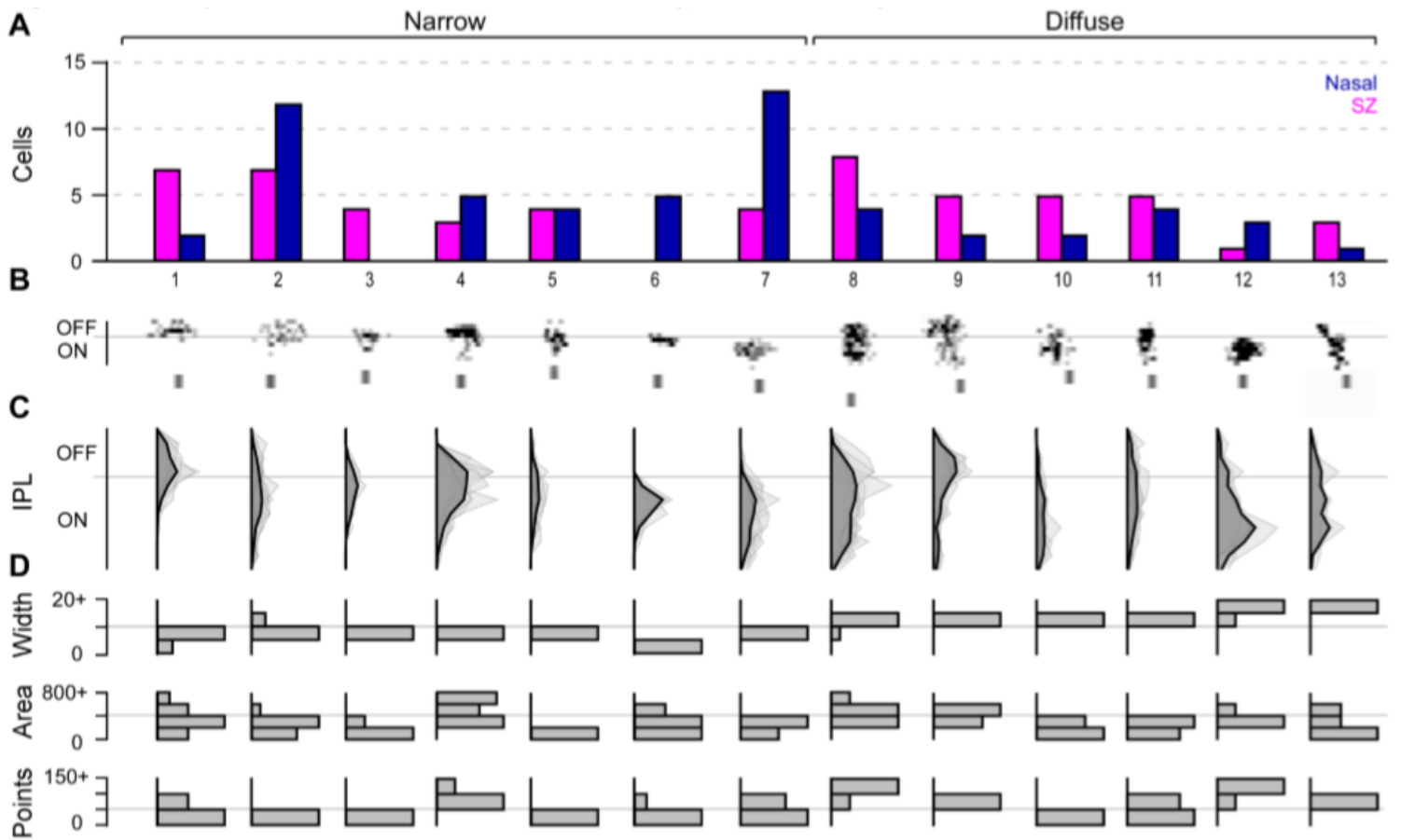


Figure 3: SZ RGCs include a relative overrepresentation of diffusely-stratifying, ON morphologies.

Photoconverted and processed RGC point clouds were projected as density maps and clustered according to anatomical criteria. **(A)** Nasal (blue) and SZ (magenta) RGC point clouds fell into 13 morphologically distinct clusters. **(B)** Sample RGC morphologies for each of the 13 clusters. Note that in this representation, each density map's IPL depth profile (y magnitude) is stretched five-fold relative to its lateral IPL stratification width (x magnitude). **(C)** Mean (dark) and individual IPL depth profiles (light). **(D)** Distribution of widths, dendritic field area, and average number of SDs per cell in each cluster.

5. CHROMATIC RESPONSES OF AZ RGCS TO FULL-FIELD STIMULI

5.1 Introduction

It is no wonder that Paley and Darwin, in sniffing through the biological world for exemplars of God's and nature's most intricate handiworks, settled upon the eye as a prime example for their agenda-pushing. Studies, dating back hundreds if not thousands of years, abound which detail the structure of the vertebrate eye: the cells and circuits comprising the retina, the bundle of nerve fibers bridging the brain's hemispheres at the optic chiasm, the dense mesh of vasculature spreading its fingers around and through the eye, the pigment epithelium shrouding the back of the photoreceptor layer like a curtain hiding cortical mysteries, and all the other tissue structures supporting the chatter of excitable visual neurons. The 20th century witnessed the expansion of investigative curiosity into new fronts as it sought to elucidate the building blocks of which the retinal machinery is comprised. The past two decades in particular have seen great headway made in the attempt to categorically uncover the genetic, transcriptomic, and proteomic constitution of retinal neurons and glia (Yang & Tezel, 2005; Rheaume et al., 2018; Schiapparelli et al., 2019). Each of these studies has contributed to the picture, slowly but steadily emerging, of the fundamental construction of the vertebrate eye.

The story of physiological investigation in the retina, by contrast, boasts no such venerable tradition dating back to antiquity. As such, the modern project to characterize function as well as form, and to show how both are two sides of the same coin used to cover the expenses of visual processing, has had significantly less by way of historical precedent to build upon. It began slowly, with electrophysiological studies in the 1950s and 1960s (Lettvin et al., 1959; Werblin & Dowling, 1969). RGCs were typically the cells of choice in these early studies, since they are the most accessible among the retinal cohort, and also carry the most highly processed signals, thus providing a direct wire tap into the type and quality of information which visual brain areas receive. The inevitable clash of classification schemes commenced as more research groups united their efforts with those of the pioneers. Different research groups working with a variety of animal models used their single-cell results to bundle the sum total of RGC responses under ON, OFF, and ON/OFF responsiveness, according to response latencies or transient or sustained voltage traces, and by the selectivity of subsets of RGCs for local motion detection, orientation, and direction preference, and excitability by uniform illumination versus those picky for contrasts of

different shapes, sizes, and colors (Lettvin et al., 1959; Barlow et al., 1964; Werblin & Dowling, 1969; Cleland et al., 1974; Derzies & Baylor, 1997). More recently, multielectrode array recordings (Farrow & Masland, 2011) and 2-photon functional imaging (Euler et al., 2002, 2009; Baden et al., 2016; Franke et al., 2017), have enabled population imaging of large sections of the retina at a time, thus at once facilitating the process of fully profiling RGCs and circumventing all the inefficiencies, technical pitfalls, and potential biases that arise through extrapolating from small, potentially handpicked sample sizes.

Here, as so often before, the full power of functional recording has only belatedly been brought to bear on the zebrafish visual system. Nevertheless, as if ashamed of its negligence and seeking to make up for lost time, what has for too long remained unexplored territory is now being explored, and at an impressive pace. The chromatic preferences of BCs (Zimmerman et al., 2018) and the particular wavelength tunings and other preferences of PRs (Yoshimatsu et al., 2019; 2020; 2021), particularly UV cones (Yoshimatsu et al., 2019; 2020), have been mapped within the past several years, providing a wealth of data on cell type- and region-specific activation profiles of outer retinal neurons. These studies have also been able to sketch a tentative line between the response profiles of different areas of the retina to the structure of the cells positioned therein, a highly telling result in itself.

Less overwhelming have been the attempts to characterize the zebrafish's innermost retinal neurons. Although recordings from ganglion cells date back to years prior to the publication of the BC and PR studies, they have been less universal in scope, constituting numerous disparate, often somewhat indirect, attempts at piecing together a full functional profile for RGCs. Extracellular recordings have confirmed that larval RGC responses include the familiar ON, OFF, ON/OFF, transient, and sustained categories (Emron et al., 2007), while optical recordings of RGC axon terminals in the pretectum and tectum have shown that ganglion cells are tuned to orientation and motion direction (Nikolau et al., 2013) and, in a subset at least, to object size (Preuss et al., 2014; Semmelhack et al., 2015). Several studies have also attempted to link broad response profiles, recorded or extrapolated from preexisting data, to RGC types in different regions of the retina (Robles et al., 2014; Semmelhack et al., 2015).

While these studies make for a promising start, they are restricted by a number of crucial limitations which preclude a complete characterization either of those RGCs inhabiting a specific

area of the GCL or of the ganglion cell population as a whole. First, attempting to characterize RGC function by recording from their arborization fields in the brain faces the significant constraint of the densely packed nature of pretectal and tectal neuropil. On account of this, such brain activity-derived extrapolations of retinal activity fail to differentiate the responses of individual RGCs, or even indeed of whatever neighboring cells the transgenic line chosen for the study may label (potentially, a high number in the case of popular lines like *elavl3/HuC*). The upshot of this is that the responses of the axonal terminals of any given RGC are not directly attributable to a single particular cell, or even a single particular region, as well as being mixed up with the response types of other RGCs and, likely, also non-retinal cells (Sajovic & Levinthal, 1983). This first point is of central import, since the zebrafish larva's large field of view eyes (Bianco et al., 2011; Patterson et al., 2013; Zimmerman et al., 2018) survey a visual space which varies dramatically from one region to another in its spatial, temporal and spectral composition as well as the behavioral relevance of features more likely to be found in some subset of these regions than in others (Engeazer et al., 2007; Zimmerman et al., 2018; Yoshimatsu et al., 2019). Accordingly, RGCs inhabiting different retinal regions are expected to maintain those— invariably different— response profiles best suited to the visual areas it surveys.

The second major drawback of these studies is technical in nature, in that it relates to the range of responses teased from the target cell population by the stimuli themselves. In the past, investigation into the function of zebrafish RGCs have made use largely of long wavelength light stimulation (Bollman, 2019), a matter which will be discussed in more detail in the subsequent chapter. At present, it merely bears to point out that this approach must miss a significant proportion of the light spectrum which the zebrafish is capable of perceiving and responding to (Krauss & Neuemeyer, 2003; Orger & Baier, 2005; Meier et al., 2018). Moreover, since particular wavelengths are associated both with different areas of the zebrafish visual environment (Zimmerman et al., 2018; Nevala & Baden, 2019) and specific behaviors (Orger & Baier, 2005; Yoshimatsu et al., 2019; Baden, 2021), including the prey capture circuit (Yoshimatsu et al., 2019; 2020), any study failing to distinguish between spectral preferences from ultraviolet to red wavelengths will be unable to capture the full chromatic, or indeed spatio-temporal, response profile of any of the retina's neuronal types. Only recently have research groups begun to capitalize on our knowledge of the zebrafish's visual environment and the spectrally diverse functions of a

retina built to accommodate its nuances (Zimmerman et al., 2018; Yoshimatsu et al., 2019; Fornetto et al., 2020; Guggiano Nilo et al., 2020; Bartel et al., 2021).

Thus, the response profile of RGCs, whether across the entire retina or in those regions associated with specific behavioral paradigms, remains a significant gap in our knowledge of retinal physiology, as does the precise correlation of particular response types to the morphological RGC types responsible for them. To begin addressing these questions, we imaged light-driven signals from AZ RGCs *in vivo*. We show that, in this region of the inner retina, diverse RGC functions exist in which UV-ON components are mixed with a variety of spectral and temporal responses in an IPL depth-dependent manner. By comparing this response profile to that of RGCs inhabiting other retinal areas, we show that AZ RGC responses are unique in terms of their high proportion of low wavelength and light-ON responsiveness. Finally, we link the spatial profiles of AZ RGC and non-AZ RGC responses to our survey of morphological heterogeneity of ganglion cell types across the retina.

5.2 Materials and methods

Animal care and transgenic lines. All procedures, conditions, and non-transgenic lines used are as described previously (See chapter 2, section 2.2: “Animal care and lines”). For all experiments, recordings were taken from either the left or right eye of 6-8 *dpf* *Tg(Islet2b:nls-trpR, tUAS:MGCamp6f)* larvae.

Two-photon calcium imaging, stimulation parameters, and light stimuli. The 2-photon microscope and stimulation parameters used for *in vivo* recording of light-driven calcium transients have been described previously (See chapter 3, section 3.2: “Two-photon imaging”). For all experiments, larvae were kept at constant illumination for at least 2 seconds after the laser scanning started before light stimuli were presented.

Three types of full-field light stimulus were used to stimulate cells in each region: 1) binary dense ‘white noise’, in which the four LEDs were flickered independently of one another in a known random binary sequence at 6.4 Hz for 258 seconds; 2) ‘chirp’ stimulus, consisting of a bright step followed by a sinusoidal intensity modulation of increasing frequency and uniform amplitude; 3) UV ‘step’ contrast stimulus, in which the RGB LEDs were held constant while the intensity of the

UV LED was increased or decreased in stepwise fashion. Recording configurations for all stimuli were 64x48 pixels at 2 ms per line. After exposing cells within the recording window to the three stimuli in the above respective order, we then moved to a new plane within the nasal or AZ.

Functional data pre-processing #1: ROI placement, quality criterion, receptive field mapping. For analyzing functional datasets, regions of interest (ROIs), corresponding to dendritic or somatic segments of RGCs were defined automatically using local image correlation over time, as described in a previous publication (Franke et al., 2017). This ROI segmentation algorithm is specifically designed to avoid merging strongly functionally distinct signals, such as a mixing of ON and OFF signals which would result in response cancellation.

As discussed in chapter 3, Islet2b labels both RGC dendrites as well as a subset of somata sufficiently to capture calcium transients. To allocate ROIs to dendritic and somatic datasets, a boundary between the GCL and IPL was drawn by hand in each scan: all ROIs with a center of mass above the boundary were considered as dendritic, and all ROIs below were considered as somatic. Only ROIs where at least one of the four spectral kernels' peak-to-peak amplitudes exceeded a minimum of ten standard deviations were kept for further analysis ($n = 2,716/2,851$ dendritic ROIs, 95%; 586/796 somatal ROIs, 74%). Equally, all individual color kernels that did not exceed 10 SDs were discarded (that is, were set to NaN).

Note that in optically-sectioned images, both those captured during periods of calcium activity and those of inactivation, mGCaMP6f expression in somata assumes a ring-like appearance in accord with the membrane surrounding the GFP-empty cytoplasmic space; therefore, it is possible that during ROI placement, multiple ROIs ended up inadvertently placed on different sections of the same soma. Since such cell body splitting was unlikely to be in any way systematic over functional types, we did not attempt to correct for this possibility. Note also that, although dendritic ROIs likely covered multiple neurites, this is not expected to have introduced any significant level of noise into the extracted responses, since the local response correlation technique we used here has been tested on signals at least as complex (Franke et al., 2017) as those of larval RGCs.

Functional data pre-processing #2: Calcium trace extraction and visual stimulus alignment. Next, the Ca^{2+} traces for each ROI were extracted and de-trended by high-pass filtering above ~ 0.1 Hz and followed by z-normalization based on the time interval 1-6 seconds at the beginning of recordings using custom-written routines under IGOR Pro 6.37 (WaveMetrics). A stimulus time

marker embedded in the recording data served to align the Ca^{2+} traces relative to the visual stimulus with a temporal precision of 1 ms. Responses to the light flash were averaged over 3-6 trials. Note that, in order to relate responses to the different stimuli to the same retinal regions, the ROI mask for the kernel stimulus was copied and used for extracting chirp and step responses.

Responses to the chirp stimulus were up-sampled to 1 kHz and averaged over 3-6 trials. For data from tetrachromatic noise stimulation, we mapped linear receptive fields of each ROI by computing the Ca^{2+} transient-triggered-average. To this end, we resampled the time-derivative of each trace to match the stimulus-alignment rate of 500 Hz and used thresholding above 0.7 standard deviations relative to the baseline noise to the times t_i at which Calcium transients occurred. We then computed the Ca^{2+} transient-triggered average stimulus, weighting each sample by the steepness of the transient:

$$F(l, \tau) = \frac{1}{M} \sum_{i=1}^M \dot{c}(t_i) S(o, t_i + \tau)$$

Here, $S(l, t)$ is the stimulus (“LED” and “time”), τ is the time lag (ranging from approx. -1,000 to 350 ms) and M is the number of Ca^{2+} events. RFs are shown in z-scores for each LED, normalized to the first 50 ms of the time-lag. To select ROIs with a non-random temporal kernel, we used all ROIs with a standard deviation of at least ten in at least one of the four spectral kernels. However, note that the precise choice of this quality criterion does not have a major effect on the results.

To categorically classify kernel polarity, we defined On and Off based on a measure of a kernel’s dominant trajectory in time as shown by its position, within the temporal stimulus sequence, of each kernel’s maximum and minimum waveform amplitude. If the maximum preceded the minimum, the kernel was classified as Off; conversely, if the minimum preceded the maximum, the kernel was defined as On.

Eye-IPL maps. To summarize average functions of RGC processes across different positions in across IPL depths, we computed two-dimensional “Eye-IPL” maps. For this, we divided the IPL into 20 bins. All soma ROIs were allocated to bin 1 independent of their depth in the GCL, while all IPL ROIs were distributed to bins 3:20 based on their relative position between the IPL boundaries. As such, bin 2 is always empty, and serves as a visual barrier between IPL and GCL. From here, the responses of ROIs within each bin were averaged. All maps were in addition

smoothed using a circular $\pi/3$ binomial (Gaussian) filter for 5% of IPL depth across the y-dimension (dendritic bins 3:20 only).

ON-OFF index (OOi). For each Eye-IPL bin, an ON-OFF index (OOi) was computed using the following equation:

$$OOi = \frac{nOn - nOff}{nOn + nOff}$$

Here, nOn and nOff correspond to the number of ON and OFF kernels in a bin, respectively. OOi ranged from 1 (all kernels ON) to -1 (all kernels OFF), with an OOi of zero denoting a bin where the number of On and Off kernels was equal.

Ternary response classification. Each ROI was allocated to one of 81 ternary response bins (three response states raised to the power of four spectral bands). One of three response-states was determined for each of four spectral kernels (red, green, blue, UV) belonging to the same ROI: On, Off or non-responding. All kernels with a peak-to-peak amplitude below ten standard deviations were considered non-responding, while the remainder was classified as either On or Off based on the sign of the largest transition in the kernel (upwards: On, downwards: Off).

Feature extraction and Clustering. Clustering was performed on two data sets, each containing the functional responses of RGCs to kernels and chirp stimuli derived from color noise stimuli: 1) the acute zone inner plexiform layer (AZ-IPL) data set (n = 3,542), sampling RGC ROIs across the AZ IPL, and; 2) the acute zone ganglion cell layer (AZ-GCL) data set (n = 1,694), sampling RGC ROIs only from the AZ RGC somata and proximal dendrites below the IPL. Mean responses to chirp stimuli were formatted as 2,499 time points (dt = 1 ms) while color kernels were formatted as 649 time points (dt = 2 ms, starting at t = -0.9735 s) per each of the four spectral color channels. Responses to UV step stimuli were analyzed separately, partitioned by means of k-means clustering, and average waveform responses compared between nasal and AZ.

For each of the two datasets, the kernels portion of the data produced a cleaner clustering than when clustering chirp responses and kernels together, or chirp responses alone; therefore, clustering was carried out using only the kernels extracted from the white noise responses. Prior to clustering, ROIs with low quality kernels, which we defined as the maximum standard deviation across the four color channels, were identified and removed from the data set. A kernel quality

threshold (peak-to-peak) of 5 was chosen, such that any ROI with a kernel quality below five standard deviations of the kernel baseline was eliminated from the data to be clustered. Following this quality control measure, the IPL and GCL datasets had the following sizes: 1) AZ-IPL: $n = 2,435$ (68.8% (3 significant figures) of the original ROI complement); 2) AZ-GCL: $n = 721$ (42.6% (3 significant figures) of the original ROI complement). We also noticed that red and green kernel populations had relatively larger amplitudes than the blue and UV kernels; therefore, in order to ensure an even weighting for each color channel in the reduced datasets, we scaled the data corresponding to each kernel color by dividing each one by the standard deviation through time and across ROIs.

In preparation for clustering, we reduced the dimensions of the two datasets by means of principal component analysis (PCA). PCA was performed using the Matlab routine **pca** (default settings). We applied PCA to those portions of either dataset which corresponded to each of the kernel colors separately, retaining the minimum number of principal components necessary to explain $\geq 99\%$ of the variance. The resulting four ‘scores’ matrices were then concatenated into a single matrix ready for clustering. The following numbers of principal components were used for each of the four data sets: 1) AZ-IPL: 15 red components, 17 green components, 25 blue components, 18 ultraviolet components (75 in total), and; 2) AZ-GCL: 20 red components, 21 green components, 27 blue components, 34 ultraviolet components (102 in total).

This combined ‘scores’ matrix was clustered by means of Gaussian Mixture Model (GMM) clustering, performed using the Matlab routine **fitgmdist**. We clustered the data into clusters of sizes 1, 2, ..., 100, using i) shared-diagonal, ii) unshared-diagonal, iii) shared-full and iv) unshared-full covariance matrices, such that 400 (100 cluster $\times$ 4 covariance matrices) different clustering options were explored in total. For each clustering option, 20 replicates were calculated, each of which had a different set of initial values; and of these, the replicate with the largest loglikelihood was selected. A regularization value of 10^{-5} was chosen to ensure that the estimated covariance matrices were positive definite, while the maximum number of iterations was set at 10^4 . All other **fitgmdist** settings were set to their default values.

In the AZ-IPL dataset, the optimum clustering was judged to be the one that minimized the Bayesian information criterion (BIC), which balances the explanatory power of the model (loglikelihood) with model complexity (number of parameters), while clusters with <10 members

were removed. In the AZ-GCL dataset, the BIC did not give a clean clustering; therefore, we manually specified 10 clusters, removing clusters with <5 members. Altogether, we obtained the following optimum number of clusters for each data set: 1) AZ-IPL: 12 clusters (1 cluster with <10 members removed), and; 2) AZ-GCL: 10 clusters (1 cluster with <10 members removed). Unshared diagonal covariances matrices gave the optimal solution in all cases.

5.3 Results

Recording light-driven responses in the inner retina. To record light-driven responses in RGCs, we performed 2-photon *in vivo* imaging on 6-8 dpf *Tg(Islet2b:trpR-nls, tUAS:MGCamp6f)* larval retinæ (**Fig. 1A**; for full discussion of this promoter-reporter system, see chapter 3). For stimulation, we used four LEDs spectrally aligned with the absorption peaks of the zebrafish's four cone opsins (Zimmerman et al., 2018) and adjusted the power according to the relative power distribution of daytime light to which zebrafish larva are exposed in their native underwater habitat (Zimmerman et al., 2018; Nevala & Baden, 2019): red (100%), green (50%), blue (13%), and UV (6%) (**Fig. 1B**). To our knowledge, this is the first time the zebrafish's 'natural white' has been used in a functional study, and it is of note that the UV and blue components of the spectral response was still substantial despite their power diminution relative to green and, especially, red.

All 2-photon images were taken in the sagittal plane from nasal and AZ, moving up and down at 5 μ m intervals. The nasal dataset was used primarily for purposes of comparison and has not been included in this chapter (but see **Supplementary Data, Fig. 2**). After zooming in on a particular region, but prior to recording, we bent the scan so as to follow the eye's curvature (**Fig. 1D**, 'banana scan', described in **Materials & Methods**; see also Janiak et al., 2019) in order: 1) to facilitate imaging only RGC somata and processes, while leaving out as much surrounding non-labeled/non-RGC tissue as possible, and; 2) to simplify subsequent analysis by using rectangular rather than curved ROI maps. For recording, we used the following full-field stimuli:

- (i) Binary dense 'white' noise, in which the four LEDs were flickered in a known random binary sequence at 6.4 Hz for 258 seconds, to assess general chromatic sensitivity. For analysis, we reverse correlated each ROI's response to the stimulus (Chichilnisky, 2001), computing a linear fluorescence response-triggered average

(commonly denoted a ‘kernel’, and visualized as a set of superimposed color-specific waveforms) of each ROI’s stimulus response properties to the four stimulating wavelength peaks. R, G, B, and UV kernels were overlaid to produce a representation of a given ROI’s response to behaviorally relevant cone-type input.

- (ii) ‘Chirp’ stimulus, consisting of a bright step followed by a sinusoidal intensity modulation of increasing frequency and constant intensity, to probe for polarity, kinetics, and temporal frequency preferences (Baden et al., 2016);
- (iii) UV ‘step’ contrast stimulus, in which the RGB LEDs were held constant while the intensity of the UV LED was changed in stepwise fashion, in order to probe for polarity and contrast sensitivity in the UV channel. The main results of note derived from applying this stimulus have to do with the difference between the nasal and AZ RGC response profiles taken as a whole. Since this chapter is primarily concerned with the AZ RGC response profile, for which the activity elicited by the UV step stimulus proved to be essentially the same as for the bright on/off step in the chirp stimulus, we postponed discussion of this stimulus’ results for the supplementary data sections accompanying this chapter (**Supplementary Data, Fig. 2**).

In a sample recording from the AZ, a rich but relatively restricted assortment of responses for GCL and IPL ROIs is in evidence. These include: 1) a preponderance of ON responses, as given by the chirp’s light-ON flashes and the polarity of the majority of kernel waveforms (decreasing followed by increasing amplitude), and; 2) a distinct short wavelength component in terms of cells’ chromatic preference, in addition to the broad R/B activation which appears to be a general feature across the larval GCL and IPL (**Fig. 1C-H**). Both these characteristics are strongly reminiscent of the functional response profiles of BCs and PRs in the AZ (Zimmerman et al., 2018; Yoshimatsu et al., 2019). Interestingly, none of these ROIs’ four color kernels display the opposing polarity characteristic of color opponency, a feature prescient of the bias toward chromatic/achromatic signaling we found, though to a limited extent, among the AZ RGC population as a whole.

AZ RGCs are ON-biased and respond best to UV light. To characterize the chromatic and color opponent computations performed by AZ RGCs, we recorded from n=87 fields of view in 28 fish at a range of depths in the sagittal plane which encompassed the entirety of the AZ. ROIs were

placed automatically on functionally homogeneous processes based on local response correlation during the noise stimulus (Franke et al., 2017). Dependent on its vertical position in the scan path, each ROI was categorized either as dendritic, if it fell within the functionally defined IPL, or somatal, if it fell within the GCL. Of the 3,542 dendritic and 1,694 somatal ROIs, 2,435 (68.7%) and 721 (42.6%), respectively, passed our quality response criteria (for full discussion, see this chapter's **Materials & methods** section). The remaining low-amplitude ROIs were discarded.

AZ ROIs were broadly classified according to the polarities and relative amplitudes of each ROI response's four color kernels: achromatic (waveforms with equal polarity and equal amplitudes), chromatic (waveforms with equal polarity but different amplitudes), or color opponent (waveforms with different polarity, or whose respective kernel waveforms were not in alignment). We first classed ROIs as either ON or OFF based on the dominant sign of their largest-amplitude kernel. Under this set of criteria, both dendritic and somatal ROIs constituted a far higher proportion of ON than OFF responses (**Fig. 2B**), in line with published BC circuits in this area (Zimmerman et al., 2018). Although this proved to be a persistent feature when extracting and comparing R/G and UV/B wavelength responses separately, this analysis separated out additional components unique to the long and short wavelength ON/OFF profiles (**Fig. 2C, left panel**). Among red and green kernels, ON responses were roughly equally divided between the two wavelengths at the level of dendrites, but not that of somata, whose green channel featured a preponderance of OFF ROIs. By contrast, the ON-bias was preserved in the red channel both in dendritic and somatal ROIs. This character was reflected in the short wavelength spectrum, in which blue kernels were strongly OFF biased while the UV channel was predominantly made up of ON responses (**Fig. 2C, right panel**).

For our second step in categorizing AZ RGC responses, we subdivided our ROIs according to the number of possible wiring motifs for combining cone-type input within the inner retinal circuit (for a full explanation of the theory underlying this approach, see this chapter's **Materials & Methods, 'ternary response classification'**, and also the figure legend for **Fig. 2D**). This revealed that the majority of RGC responses fell into functional subsets represented by relatively simple wiring diagrams, most of which were ON and comprised a UV component in addition to some combination of R, G, and B (**Fig. 2D**). This is indicative of the trend in the AZ, among both BCs and PRs, toward UV-ON responses piggybacking on more generic circuits present across the retina (Zimmerman et al., 2018; Yoshimatsu et al., 2019), such that most kernels were categorizable by

their proportion of UV-to-RGB sensitivity. That three of the most common combinations were RGBUV_{ON} , RGUV_{ON} , and RBUV_{ON} (**Fig. 2E**) would seem to confirm this notion, as well as to usher AZ ganglion cells under the purview of the outer retina's known physiological construction in terms of spectral diversity and ON/OFF index. Indeed, only a small fraction of ROIs constituted OFF responses, of which most had a strong R or R/G component, often with little or no activation at the UV end of the spectrum.

Our data also uncovered numerous color opponent computations among AZ RGC ROIs (**Fig. 2D**). Of these, many had a single zero crossing in wavelength, where a change in the sign of the function—either from positive to negative or vice versa—represented by the intercept of the axis, denotes a neural computation between two color response profiles (i.e., Red ON combined with Blue OFF). More than half, however, described more complex opponencies with more than one zero crossing (i.e., Red ON, Green OFF, and Blue ON again), suggesting that different spectral alignments are a crucial feature of larval zebrafish color vision. It is interesting to note that, among the color opponent bins, several of the most frequently encountered signals were driven largely by a sluggish blue OFF component. This signal, without which the vast majority of AZ RGCs would be classified as non-opponent ON cells, appears to be pervasive across the entire retina (data not shown).

The temporo-spatial organization of AZ RGCs. Including ON/OFF indices and opponency probabilities, we extracted more than a dozen components per spectral kernel, in order to cluster based on a wider range of response properties than opponency alone. To systematically assess how our chirp and kernel responses are distributed in space across the AZ and in time, we mapped each dendritic and somatal ROI to a bin within an 'Eye-IPL map' (**Fig. 3A-K**). In this scheme, the x-coordinate represents position across the eye from the temporal horizon (bin #1) and AZ (bin #2) to ventral (bin #8), dependent on which of the eight circumscribed regions from which each ROI was acquired in the sagittal plane. Bins #1 and #2-8 are of interest to this study insofar as they were used for purposes of comparison; however, because the dataset and accompanying attempts at explanation presented in this chapter includes those responses extracted from AZ ROIs, only bin #2 is shown containing data. Within this AZ-inclusive dataset, the y-coordinate represents IPL depth, with upper rows representing laminae closer to the INL and lower rows those in the proximity of the GCL: the brighter the row, the greater the number of ROIs falling into that level

of the IPL. GCL data, analyzed separately as in the previous section, was connoted by demarcation at the base of the IPL; since it is difficult to divide a cell body layer into subregions, this second dataset was not analyzed in a layer-by-layer manner as were dendritic ROIs, but only according to their temporo-functional divisions.

By computing the AZ's mean light response to the noise and chirp stimuli and plotting them in a depth-dependent manner, we obtained a functional map showing the mean AZ RGC response in terms of contrast, kinetics, and color over time at either a given IPL depth or within the GCL. We then identified the major functional groups present across the inner retina, using a Mixture of Gaussian model (MGM) to sort functional RGC types according to their full temporo-chromatic response profiles.

Dendritic ROIs fell into $n=13$ clusters, of which 12 containing ten or more members were retained for further analysis. Likewise, of the $n=10$ clusters into which somatal ROIs were subdivided, 9 were retained. Among both IPL and GCL, the striking ON-bias across all wavelengths and the preponderance of UV responses already remarked upon were apparent (**Fig. 3A-K**). The individual response types of which the overall AZ IPL functional profile was comprised, however, were not homogeneously present across the plexiform layer, but were instead allocated to particular sublaminae, showing a wide range both in terms of relative depth in the IPL and spread within the preferred regions (**Fig. 3A-G**). For example, the clusters exhibiting the strongest UV response, C_{1-2} , fell almost exclusively into IPL rows #11-20 representing the lower IPL strata. In fact, every cluster with a prominent UV component was represented within the ON IPL laminae, and were often smeared out across the lower three-quarters of the IPL. This broad preference for ON IPL laminae to the exclusion of OFF layers (**Fig. 3C**) is reminiscent of the diffuse arborization patterns we have shown to characterize the largest fraction of AZ RGC dendritic morphologies. Also, in alignment with our AZ paGFP dataset, C_{12} , the sole cluster whose ROIs inhabited only one or two IPL sublaminae, was restricted exclusively to the OFF IPL. This depth profile, constituting just 5% of all ROIs, is suggestive of the narrowly-stratifying AZ morphological phenotype which selectively innervates IPL laminae bordering the INL. Tellingly, this single OFF cluster was long-wavelength biased, and sported one of the smallest-amplitude UV kernels of all the clusters.

Our paGFP data in non-AZ retinal regions suggests that the relatively high proportion of broadly- to narrowly-stratifying dendritic arbors is unique to the AZ. If so, this should be realized in varying

depth profiles among the functional responses characteristic of the different retinal regions, with the temporal IPL exhibiting a more diffuse functional profile for individual response types than those in nasal, ventral, or dorsal, all three of which are regions dominated by monostратified RGCs (see chapter 4). As discussed above, this does appear to be the case for the AZ response profile. In comparison to the functional profile of the retinal average, moreover, AZ RGC dendritic responses stand out for the manner in which functionally-distinct clusters tend to be distributed across large sections of the IPL, while the minority which do not share this characteristically distinguishing feature are relegated to innervating the thin OFF IPL (**Fig. 3C, I**). Across all retinal regions, the decreased number of functional clusters whose ROIs span broad sections of the IPL is matched by a relative increase in those inhabiting thin sections of either the ON or OFF IPL (**Fig. 3L**), in stark contradistinction to that of the AZ (**Fig. 3M**).

Relatively slowed central frequency tuning in the AZ. An additional intriguing feature of the AZ RGC temporal response profile was its relative slowness in comparison to the rest of the eye. Within the ventrotemporal acute zone, ON and OFF kernels maintained a relatively stable spectral centroid across all four color channels (**Fig. 4A-B**). However, with respect to the retinal average for kernel responses in the UV channel, AZ UV signals exhibited a distinct shift toward lower-frequency, and thus longer-duration, responses (**Fig. 4C-D**). This was not the case for the other three wavelength channels; nevertheless, since UV signals are more abundant in the AZ than elsewhere in the retina, this had the effect of subtly attenuating the overall integration time of AZ light signals. The blue response may also contribute to the AZ's relatively slow response profile, but since this component appears to be a general feature of RGC activity across the inner retina, it cannot be considered AZ-specific.

5.4 Discussion

In the present chapter, we have described the functional profile of AZ RGCs in terms of its chromatic, temporal, and spatial arrangement. We have shown that this population of ganglion cells is characterized by a diverse collection of ON-predominant circuits featuring combinations of short and long wavelength preferences which, while being spectrally broad, maintained a pronounced UV bias. This is similar to that of AZ BCs and PRs (Zimmerman et al., 2018; Yoshimatsu et al., 2019), confirming the notion that the AZ serves to siphon out UV-positive

aspects of the upper-frontal visual scene and to preferentially transmit these to midbrain visual structures. Moreover, this UV response was markedly slower than in the rest of the eye, mirroring the prolonged integration times in AZ UV cones (Yoshimatsu et al., 2019). Finally, we have linked the IPL response profile of AZ RGCs to our ganglion cell stratification data and shown how the location of particular response types within the AZ IPL is not the same as in other areas of the retina. Altogether, our results strongly support the idea that the larval zebrafish *area temporalis* is specialized for capturing, integrating, and transmitting small-field, short wavelength-biased information to pretectal and tectal arborization fields, that the RGC physiology which supports this function has its roots in the anatomical construction of the AZ, and that the AZ's functional as well as its structural profile is unique with respect to the rest of the larval retina.

With all of this in mind, what new conclusions can be drawn about the specific relation between RGC function in the AZ and prey capture? How well does a short wavelength-biased inner retinal circuit in the AZ meet the demands of the larva's hunting behavior as we currently understand them, and what prey capture-related features of visual perception does our functional map actually explain? Finally, with regard to the spectral and temporal diversity of AZ circuits, what potentially non prey capture-related visual tasks might they perform whose necessity is such as to demand a large share in the resources of a retinal area whose primary postulated function is of such high biological and behavioral import?

A diversity of long and short wavelength biased ON circuits to match the visual statistics of a freshwater environment. The broad spectral response profile of AZ RGCs comprised a diversity of diffusely-stratifying ON circuits, which covered the extreme short end of the visible spectrum as well as the more common long wavelength ones. If this were all the species-specific data we had from which to draw conclusions, it might at first appear puzzling as to why it would be beneficial for any part of the eye to invest so heavily in short wavelength circuitry when the UV and blue components represent such a small fraction of the zebrafish habitat's light content (Zimmerman et al., 2018). The answer, as we have been at lengths to describe throughout this report, is presumed to lie in the relative distribution of short, middle, and long wavelength photons across the larva's visual field, and on the relevance of particular spectral frequencies to specific behavioral needs.

Underwater, the UV signal is relatively stronger toward the surface and grows rapidly more attenuated at increasing depth (Zimmerman et al., 2018). Because water is so effective at scattering UV light, it creates a homogeneous background (Janssen, 1981) which has been hypothesized to serve as a spotlight against which achromatic silhouettes and strongly UV reflective objects become visible (**Fig. 5B**; Loew et al., 1993; Browman et al., 1994; Losey et al., 1999; Zimmerman et al., 2018; Yoshimatsu et al., 2019). In the case of the aquatic microorganisms which zebrafish larvae prey upon in experimental settings, and which selectively scatter UV as opposed to longer wavelength light (**Fig. 4C-D**; Johnsen & Widder, 2001; Yoshimatsu et al., 2019), circuits designed to detect such UV bright prey objects would be expected to compensate for the scarcity of environmental UV photons by investing in one or more physiological mechanisms designed to increase the system's gain. Our results indicate three cell- and circuit-level investments the retina makes in order to accomplish this.

The most mechanistically straightforward means by which the AZ could increase the gain of this subset of signals would be to increase the ventrotemporal retina's complement of short wavelength responsive circuits relative to midrange or red ones, thus improving UV photon catch. As indicated in the preceding sections, our data provide ample evidence that the inner retina utilizes this option. AZ RGCs appear to preferentially select from among BC and AC inputs for those spectral signals which contain a UV component, thus building on the UV monochromatic and spectrally mixed circuits present in earlier retinal layers (Zimmerman et al., 2018; Yoshimatsu et al., 2019).

A second means of streamlining the short wavelength information highway would be to increase the signal-to-noise ratio of UV signals, perhaps by designing, for example, circuits which up-sample particularly strong UV signals relative to weaker UV or non-UV inputs. This is one possible reason for the relative overrepresentation of diffuse dendritic stratifications among ON RGCs in the AZ. That is, by allowing individual cells to integrate signals from a broad range of chromatic circuits describing the upper-frontal visual field, they might plausibly increase the signal-to-noise ratio and thus amplify small-field UV signals sufficiently to allow prey detection. In addition, the relatively high proportion of RGCs which fall within this morphological class suggests that this AZ-specific phenotype might contribute to whole-field UV sensitivity.

The third method— and the main one likely being utilized— would be to increase the duration of the AZ circuit's response to UV stimuli. Indeed, the sluggishness of the AZ's short wavelength

response compared to the retinal average is perhaps the most clear-cut of the results we have presented in this chapter, and it parallels the kinetics of the AZ UV cone response (Yoshimatsu et al., 2019). A slow recovery to baseline calcium activity effectively prolongs the integration time of short wavelength signals and increases the time during which inner retinal cells can accumulate and process presynaptic input, thus supporting prey capture by increasing the chance that UV bright objects such as paramecia will trigger retinotectal action potentials. Ultimately, of course, this allows the larva sufficient time to detect and react to prey objects. In addition, however, the fact that this attenuated response time is strictly a feature of AZ cells may ensure that only potential prey objects present in the upper-frontal visual field are capable of activating prey capture circuits, allowing the hunter to focus on foodstuffs in this area of visual space by minimizing distraction from peripheral UV photons.

But what purpose is served by the AZ's high proportion of RGB as well as UV input? The prey capture paradigm provides a tentative reason for why the AZ invests so heavily in UV circuitry, as well as explaining other features of retinal anatomy and function which are specific to the *area temporalis*, but not why the UV monochromatic system appears to be superimposed on top of long wavelength circuits common to the rest of the larval retina (Zimmerman et al., 2018; Yoshimatsu et al., 2019). It is unlikely that the green and red channels play an integral role in the detection of prey objects, since aquatic zooplankton are largely transparent at these wavelengths (Johnsen & Widder, 2001; Yoshimatsu et al., 2019). So, why devote precious space and metabolic resources to them in an area burdened with supporting an essential biological need, especially when these circuits already exist elsewhere in the eye?

Although there are several ways of considering this question, and therefore of answering it, the hypothesis with the most obvious explanatory power is that the importance of the UV circuit does not obviate the need for other behaviorally relevant spectral computations to take place in a given retinal area, even one as critical as that for prey detection and pursuit. Furthermore, unlike UV and, to a lesser extent, blue light, the long wavelength light content of freshwater systems tends to be stronger at all depths, and more uniformly absorbed, creating very different visual field inputs in the green and red channels than for the other two (Losey et al., 1998; Zimmerman et al., 2018). Simple pixel detection and image formatting, therefore, would require a contribution from long wavelength systems across the visual field. In addition, the AZ circuits, including but not restricted

to the UV channel, have been proposed to contribute to the detection of approaching predators, which might appear as dark contrasts in the upper frontal visual area (Cronin & Bok, 2016; Zimmerman et al., 2018). This may be facilitated by using achromatic rather than merely monochromatic circuits, particularly since the red, green, and blue channels appear capable of picking up dominant spatial features of visual scenes which the blurry UV channel cannot (Zimmerman et al., 2018; Nevala & Baden, 2019). This is an aspect of underwater visual statistics about which we will have more to say in the next section with respect to the larval retina's color opponent system, as well as in subsequent chapters when describing our behavioral experiments.

Color opponent computations in the AZ. In attempting to explain how color perception taking place at the receptor stage can yield vision at the neural-computational level, opponent process theory posits complex interactions of suppression and activation between different color channel groupings at successive stages within the network—although in a more technical meaning it refers to any comparison in tuning, and in reality would comprise a mix of spatial, spectral, and temporal components. But whether used in the broader or more restricted sense of the term, the notion of wavelength ‘opponency’ is assumed to be a universal feature of color vision (Baden & Osorio, 2019), having been extended from its original 19th century application within trichromatic systems to draw inferences about tetrachromatic vision (Neumeyer, 1992; Dorr & Neumeyer, 2000; Krauss & Neumeyer, 2003), including that of the zebrafish (Meier et al., 2018).

As described in this chapter's results section, most of our somatal and dendritic RGC responses fell into functional subsets whose wiring motifs are relatively simple compared to the number and types of RGBUV and ON/OFF combinations (**Fig. 2D**), in accordance with previous experimental findings and theoretical accounts which suggest that parsimony is the rule in visual system construction (Kamermans et al., 1991, 1998; Zimmerman et al., 2018; Baden & Osorio, 2019). Moreover, the many color opponent computations performed by AZ RGCs share the distinctive UV-ON bias which is already evident in BC terminal signals within this region. However, whereas the former cell population seems to perform additional spectral computations and in general incorporates a greater degree of variety in their tuning, the latter interneurons were more simply characterizable as UV-monochromatic— even though color opponent clusters, both those with and those without a significant short wavelength component, are present in BC terminals surveying the lower and middle visual field (Zimmerman et al., 2018). What may be added here is that, given

the anisotropic functional profile of BCs across the eye, and of the increasing complexity of AZ RGC responses relative to their presynaptic counterparts, it is quite likely that other and more diverse color opponent RGC responses are present in different retinal regions, within both the GCL and IPL.

All of this is well in line with the predominance of simple over complex contrasts in natural scenery (Buchsbaum & Gottschalk, 1983; Maloney, 1986; Ruderman et al., 1998; Lewis & Zhaoping, 2006; Zimmerman et al., 2018; Nevala & Baden, 2019). It also fits nicely with a commonly-held view in visual research that image forming vision is the sole or primary purpose of spectral and temporal processing with regard to opponency (Baden et al., 2019). Yet, as we have endeavored to show in our discussions up to this point, this need not be the case. As far as the AZ's complement of RGC color computations go, its distribution far outnumbers both the spectral and temporal computations, at least, of BCs: spectral, in the number of wiring patterns recorded; and temporal, in that their spectral kernels were often asymmetric mixes of long and short wavelengths with unaligned peaks. Of course, some increase in signal diversity is to be expected between BCs and RGCs. But with almost half of all retinotectal output channels classifiable as color opponent, the real question of interest is why the zebrafish larva's visual system is not *more* parsimonious. It is possible, too, that our dendritic ROIs may have merged signals from two or more RGC processes, since some dendrites might have been smaller than our standard 3pixel-by-3pixel ROI size, so that, if anything, the real diversity is likely to be higher than that presented in **Figs. 1-3**.

A possible explanation for this increased expenditure toward signal diversity follows to a large extent from the results and conclusions of the previous section. In summary form, this is the hypothesis that there may be an output channel specific to a given region— in this instance, the predator- and prey-responsive AZ— which builds on or supplements, rather than merely extending, a more general and ubiquitous channel— what we have in mind here being that for color vision, and which is typically imputed to model organisms of visual system function. If this is the case, as our recording data seem to imply, then these AZ channels may operate in parallel, with one employing long wavelengths in a manner more or less common across the retinal hemisphere, and the other, short wavelength-tuned channel used to detect UV-bright or UV-dark foreground objects against the ultraviolet haze of the upper frontal visual field (Yoshimatsu et al., 2019).

The relevance of this particular point can be best illuminated by noting that this is not to say that there need be only two channels. For example, as an explanation more pertinent to R, G, and UV responses, it potentially leaves available the blue channel, several interesting characteristics of which have already been remarked upon in this chapter. As odd color out, it might contribute to one or both of the long and short wavelength specific channels, or it might operate solo toward some other, more exclusive purpose. This could be a very interesting and fruitful line of enquiry into color channel systems with apparently ‘spare’ PR types, which might be capable of segregating short, medium, and long wavelength circuits toward serving different functions, and which might also help to explain the inner retina’s network complexity, even while few of the individual circuits are themselves complex.

But whatever the number of functionally distinguishable channels present in the larval zebrafish retina, it still remains to be asked to what extent the physiological profile maps onto behaviorally relevant perception. For the AZ in particular, what chromatic signals, attributable to which of the proposed channels, are the cells in that region capable of responding to sufficient to produce motoric responses? Do chromatically distinct stimuli elicit distinct patterns of response, and how are these related to hunting routines? To a preliminary attempt to answer such questions we turn in the next chapter.

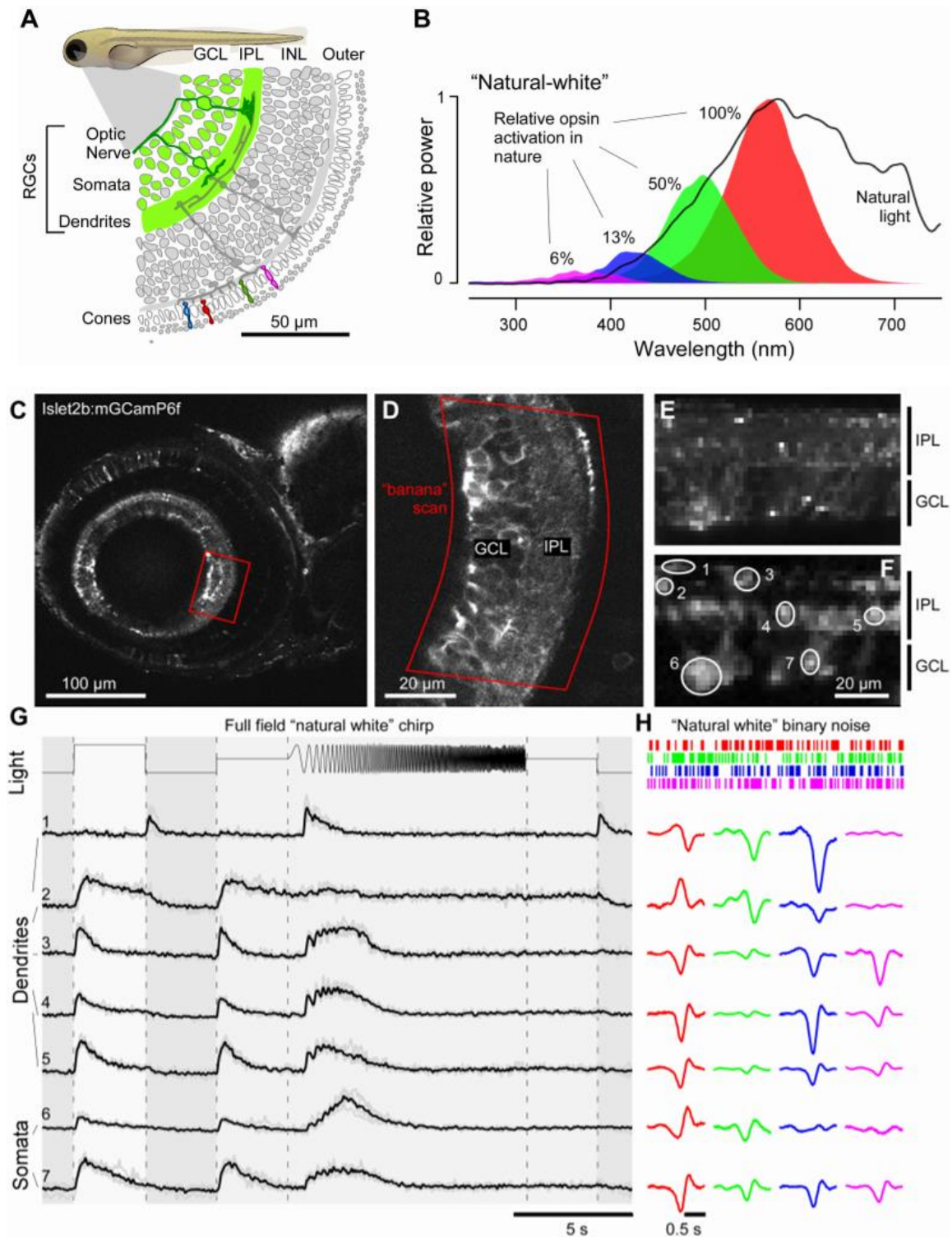


Figure 1: Overview for SZ RGC dendritic and somatal recording.

(A) Schematic of *Tg(Islet2b:nls-trpR, tUAS:MGCaMP6f)* larva, showing *Islet2b* expression in SZ RGC somata and dendrites. (B) Average daytime light spectrum (absorption) along the zebrafish larva's underwater horizon (solid black line), used to set the relative power distribution of the four stimulating LEDs to the relative power each cone surveys in nature, normalized to red cones (100%). (C) *Islet2b:nls-trpR, tUAS:MGCaMP6f* expression in the sagittal plane, as seen under our 2-photon microscope. (D) Zoom-in on the SZ for functional recording, including the curved 'banana scan' path which was straightened prior to recording. (E-F) Sample straightened activity scan (E) and activity correlation projection (F) after playing the white noise stimulus, with sample ROIs highlighted for analysis. (G) Individual calcium indicator traces (gray) and mean (black) calcium transient traces of the ROIs shown in (E) to the chirp stimulus. (H) Linear kernels extracted by reverse correlation from calcium traces from ROIs in (E) to R, G, B, and UV LEDs. Note that the B and UV responses are comparable in amplitude to the long wavelength components, despite their 8-fold (B) and 19-fold (UV) attenuation relative to the R power setting.

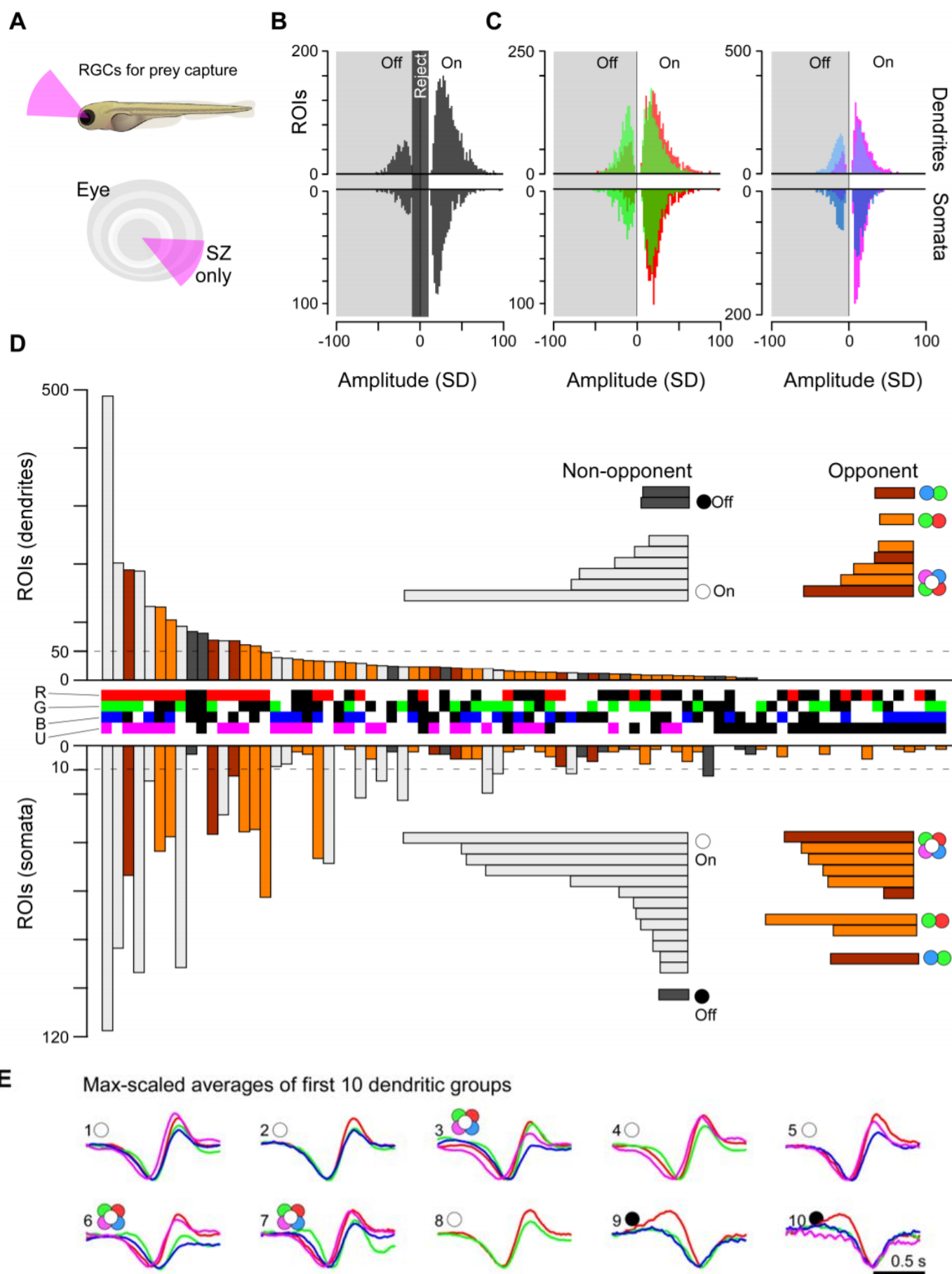
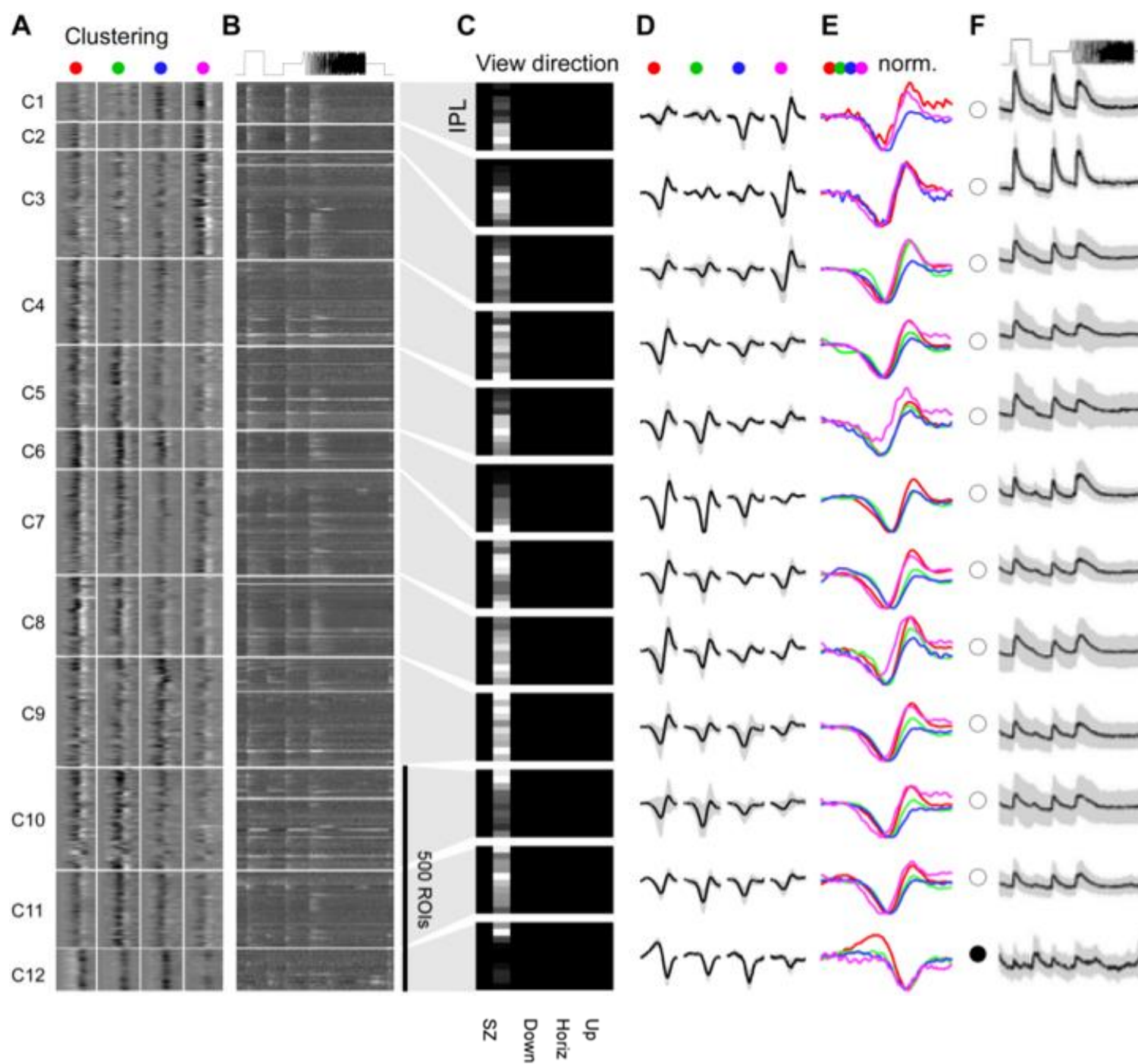


Figure 2: Color-based response profiles of RGC dendrites and somata.

(A) The ROIs comprising this functional dataset were taken in the ventrotemporal retina. **(B)** Overview of dominant OFF and ON responses in dendritic (upper) and somatal (lower) ROIs. **(C)** Breakdown of dominant OFF and ON responses according to long (left) and short wavelength (right). **(D)** Ternary spectral classification of SZ dendritic (upper) and somatal (lower, inverted y-axis) ROIs according to the possible wiring motifs for cone-type input. This is calculated as the number of possible wiring states ('ON', 'OFF', 'no connection') raised to the power of the number of cone types. For the tetrachromatic zebrafish retina, possessing four cone photoreceptors, there are a total of $3^4 = 81$ potentially-realizable wiring combinations among RGCs: 50 which are color opponent, 15 which are non-opponent ON, 15 which are non-opponent OFF, and 1 for which no cones are functionally connected. The central row between the dendritic and somatal bar graphs indicates each bin's spectral profile, listed in order of their representation among inner retinal responses and with each column showing their response to stimulation by the corresponding wavelength: 'ON' (R, G, B, and UV blocks), 'OFF' (black blocks), and 'unresponsive' (white blocks). For example, the leftmost group contains ROIs with ON kernels in all four wavelength channels, while the group second to the left comprises ROIs with ON kernels for R, G, and B but no response in the UV channel. The bar graphs, representing the relative proportion of ROIs within each bin, are color coded as follows: dark grey (non-opponent OFF), light grey (non-opponent ON), brown (opponent due solely to B OFF component), and orange (all opponent responses not containing a B OFF component). Dotted horizontal lines indicate the threshold for the minimum number of ROIs a bin needed to have in order to be included in the horizontally oriented summary bars above (dendritic) and below (somatal) the chart. For brown and orange summary bars, the colored circles to the right of each bar give its main spectral computation. Two-colored symbols denote 'simple' opponencies, consisting of single spectral zero crossings (such as red versus green) between the two wavebands indicated by the colors, while the four-colored symbols indicate complex opponencies, featuring more than one zero crossing (such as red and green versus UV). **(E)** Maximum amplitude-scaled average kernels from dendritic ROIs of the ten most frequently recurring spectral responses according to class in **(E)**.



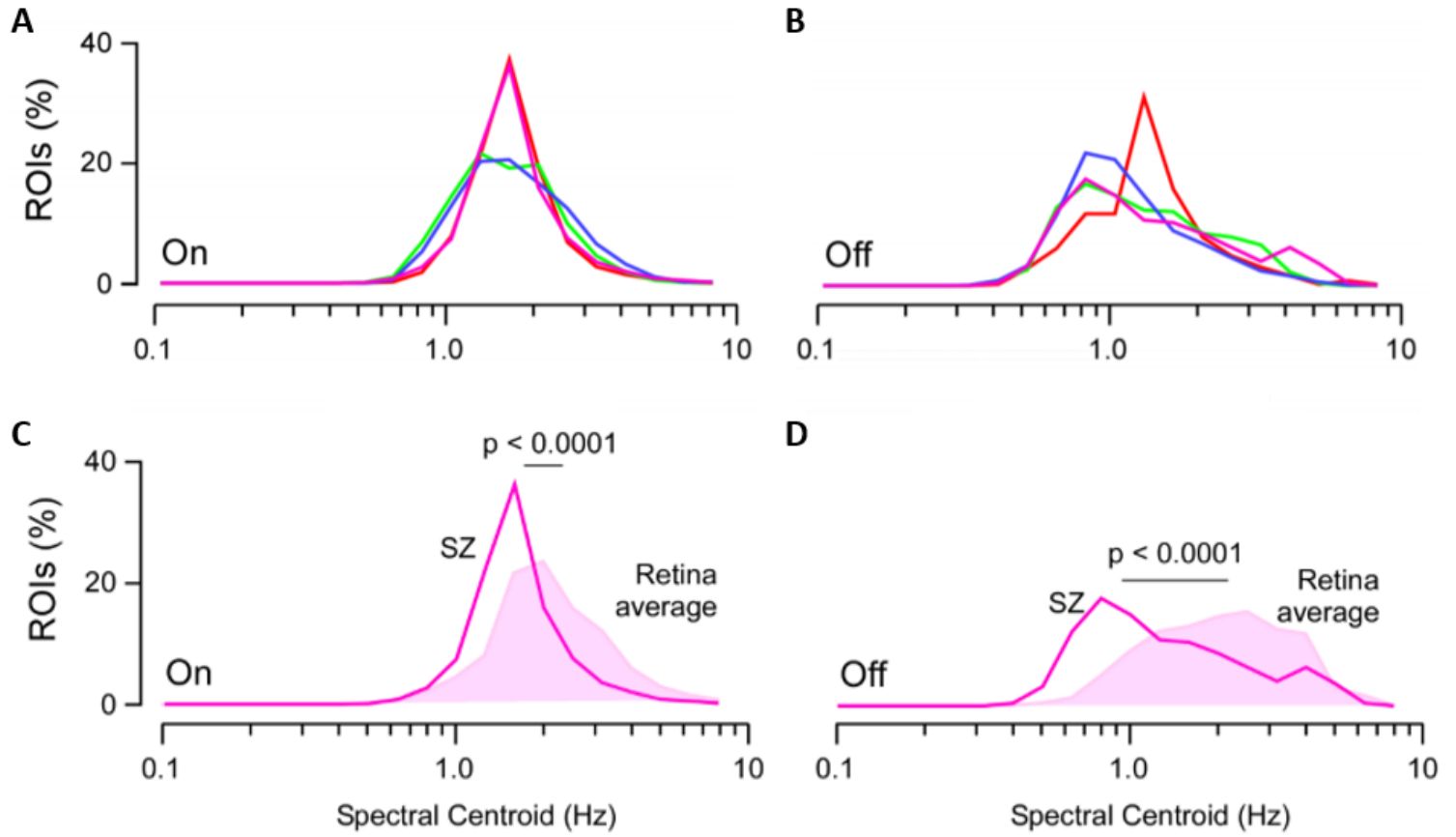


Figure 4: The UV component of SZ RGC responses is slow.

(A-B) Response frequency curves for R, G, B, and UV channels for ON (A) and OFF (B) kernels in the AZ. Note the relative similarity of the distributions, and in particular the spectral centroid (where the frequencies of most of the measured responses cluster), for the four color channels, with the exception of the R OFF response. (C-D) Response frequency curves of ON (C) and OFF (D) UV kernels in the AZ (solid purple lines) compared to the retina average of UV kernels across the eye (shaded purple regions). The difference between AZ and whole-retina spectral centroid for both ON and OFF kernels was highly significant (both $P < 0.0001$, one-tailed Wilcoxin Rank Sum test).

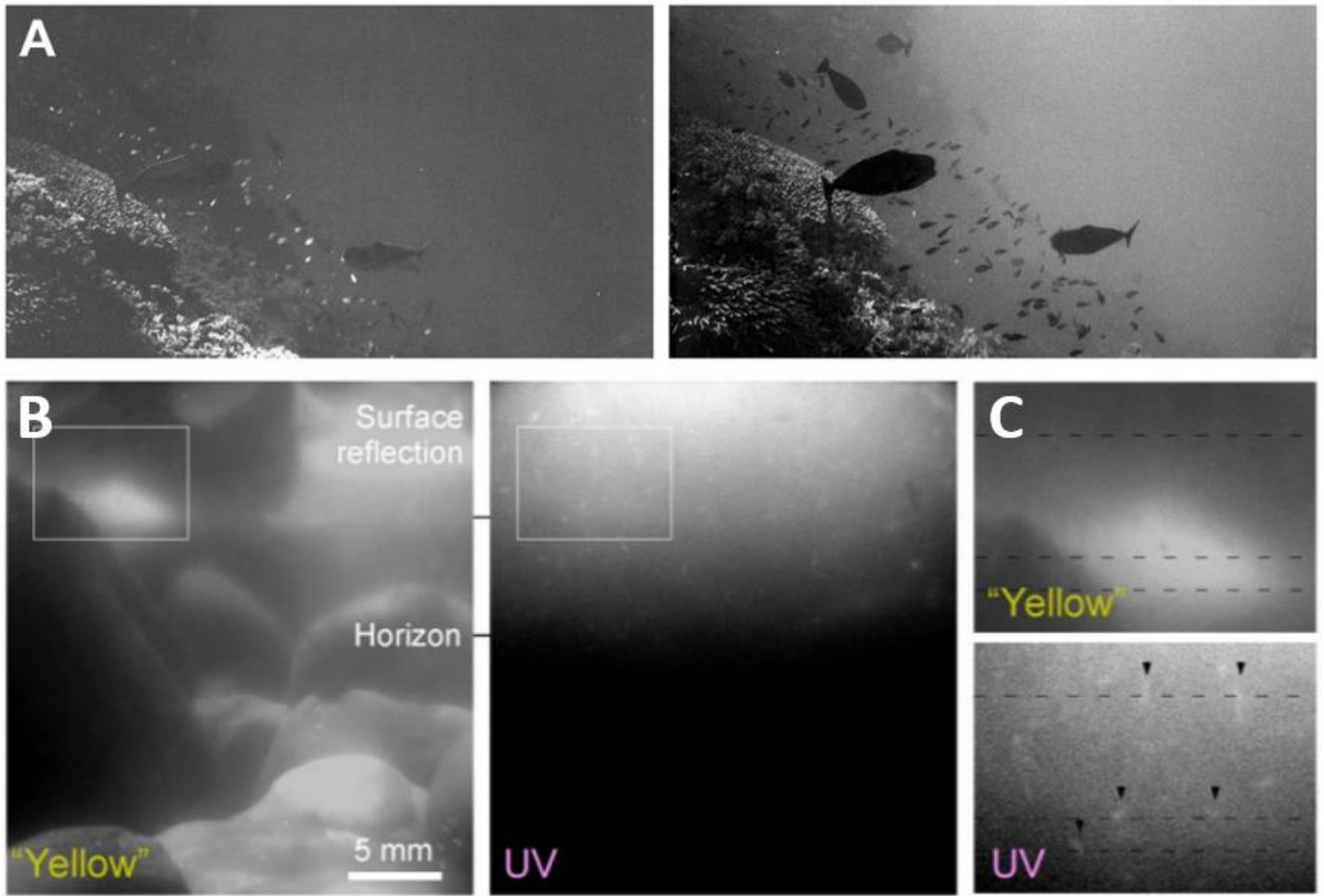


Figure 5: Predator and prey detection in natural scenes using UV light.

(A) Coral reef scene taken through bandpass filters for green (left) and UV (right) light, to simulate the view as seen at wavelengths visible to trichromatic humans and tetrachromatic fish, respectively. Adapted from Cronin & Bok, 2016. (B-C) Similar to (A), images taken to image paramecia using yellow (left) and UV (right) bandpass filters (B) and zoom in for the yellow (upper) and UV channels (lower), with arrows indicating paramecia (C). Adapted from Yoshimatsu et al., 2019.

6. BEHAVIORAL RESPONSES TO SPATIAL STIMULI AT A VARIETY OF WAVELENGTHS

6.1 Introduction

In attempting to understand vision in natural systems, anatomical and physiological studies have sought to determine the structure, organization, and function of the zebrafish larva's ocular apparatus at the level of cell and tissue and complete organ. By contrast, a more strictly ecological approach will address itself toward interpreting elements of the teleost's physical surroundings, both organic and inorganic, which exert their biological pressures upon the larva as it attempts to satisfy the perennial impulses of survival and reproduction.

Where these two oppositely originating methods of research meet, and the final crucial clue to connecting eye and environment, lies in the animal's behaviors, shaped as they are by instinct, necessity, and external circumstance, and guided by a physical constitution adapted over innumerable generations. By measuring context-dependent patterns of motor activity in response to experimentally controlled stimuli, a first step may be taken toward understanding the match between visuomotor pathways and the relevant features of real-world environments which these neural information highways transmit. In the case of the zebrafish larva prey capture paradigm, such an approach would be a means of determining whether the perception of properly prey-like stimuli by a visual system which is heavily invested in prey feature-sensitive retinal circuits preferentially elicits behaviors which correspond, if only in kind, to the type and degree of that neural investment.

The larval zebrafish AZ appears highly adapted to detecting small, rapid onset (Yoshimatsu et al., 2019), fast-moving objects (Semmelhack et al., 2015; Yoshimatsu et al., 2019) displaying at the short-wavelength end of the electromagnetic spectrum (Zimmerman et al., 2018; Yoshimatsu et al., 2019; Mingyi, Bear, et al., 2020), all of which are characteristics of the aquatic microorganisms preyed upon by larvae (Novales Flamarique, 2012, 2016; Zimmerman et al., 2018; Yoshimatsu et al., 2019). As detailed in the preceding chapter, the most straightforward hypothesis by which to explain this coincidence of capacity and behavioral requirement gives that visual cues presenting a complex of such features— both live paramecia and their computer-generated counterparts— should motivate actions associated with tracking and hunting.

However, although numerous studies have been undertaken to document larval eye and tail kinematics in response to prey-like stimuli (Budick & O'Malley, 2000; Borla et al., 2002; Hernandez et al., 2000, 2002; McClenahan et al., 2012; Patterson et al., 2013; Trivedi & Bollman, 2013; Marques et al., 2018; Mearns et al., 2019), none have sought to parse out the contribution of individual color components of the stimuli by assessing the relative weight of short- versus long-wavelength light in producing the larva's stereotypical hunting sequence. In characterizing that sequence, many studies have employed live paramecia, which trigger hunting behavior (Hernandez et al., 2002; McElligott & O'Malley, 2005; Patterson et al., 2013; Trivedi & Bollman, 2013; Semmelhack et al., 2015; Marquez et al., 2018; Mearns et al., 2019) but offer only limited means of elucidating the specific chromatic mechanisms enabling prey detection and pursuit. Conversely, virtual hunting assays endeavoring to fine tune the parameters of 'moving dot' stimuli— which recreate aspects of prey objects as a motion picture— have focused primarily on shape, size (Bianco et al., 2011), or speed (Semmelhack et al., 2015) while paying little attention to color content, contrast, or distribution. Moreover, studies using such virtual prey have relied on black (Antinucci et al., 2019), red (Bianco & Engert, 2015; Antinucci et al., 2019), or 'white' stimuli (Bianco et al., 2011; Semmelhack et al., 2015). To the best of our knowledge, none have included a UV component, with the result that even the 'white' stimuli would not appear true white for the tetrachromatic zebrafish.

Thus, despite the wealth of converging evidence for a dedicated UV prey detection pathway, the behavioral relevance to prey capture routines of short wavelengths remains to be confirmed, as by extension does the relevance of the retina's complement of UV responsive retinal cells. To begin assessing the contributions of UV to the larval zebrafish's perception of prey-like objects, we performed behavioral recordings of head-fixed larvae presented with moving stimuli given at single wavelengths corresponding to the zebrafish cone sensitivities, and compared the eye and tail responses generated by each of the four color channels. By ascertaining the relative effectiveness in initiating hunting routines of UV-bright prey-like objects, compared to those presenting at longer wavelengths, we provide a baseline verification of the proposed link between the biology of the larvae's UV-ON circuit endowment and their behavior in response to the principal chromatic components of environmentally relevant visual input.

6.2 Materials and methods

Animal care and transgenic lines. All procedures, conditions, and non-transgenic lines used are as described previously (See chapter 2, section 2.2: “Animal care and lines”). All experiments were performed under approval from the Hong Kong University of Science and Technology’s Animal Studies Committee.

Spatial stimulator and stimulation parameters. For all *in vivo* behavioral experiments, we used a custom-built arbitrary-spectrum spatial visual stimulator (designed by K. Franke and M. Chagas (Franke et al., 2019); purchased through E.K.B. Technologies) consisting of a DLP LightCrafter 4500 (referred to hereafter as an ‘LCr’; developed by Texas Instruments (Dallas, TX)) fitted with four customized LEDs and filter combinations adjusted to the wavelength peaks of daytime light in the zebrafish natural habitat (Zimmerman et al., 2018; Nevala & Baden, 2019). Because a single LCr can power a maximum of three LEDs at a time, we did not have the capacity to drive all four simultaneously to simulate the zebrafish’s natural white, but restricted ourselves to activating either individual color channels (UV, B, G, or R) or combinations of two color channels (UV/B, G/R).

Visual stimuli and experimental setup. Visual stimuli were designed using the Python-based, open-source visual stimulation software QDSpy (documentation: <http://qdspy.eulerlab.de/>). Since larvae have been shown to be sensitive to both size and speed of the prey-like stimulus, our basic stimulus consisted of a small moving dot, covering approximately 3° of the larval zebrafish visual field and traveling horizontally from left to right at 90°/s against a black background (adapted from Semmelhack et al., 2015, who characterized an ‘optimal stimulus’ for evoking hunting movements in terms of size and speed). This moving dot stimulus was presented in each of the four color channels individually, and was comprised at a given time of a single-wavelength-channel sweep: for example, a red spot on black a background, followed by a spot of a different color on the same black background, and so forth, until each of the four wavelengths had been presented (**Fig. 1A**). The stimuli were projected onto a flat Teflon screen covering one side of a 4inch-by-4inch water-filled aquarium.

In preparation for behavioral recordings, we embedded 5-6 *dpf* larvae 3cm from the edge of a 100cm Petri dish, one-sixth of the perimeter of whose walls were cut back so as to permit its being fitted against the aquarium’s viewing screen. After the agarose had been allowed to set, we cut

away the agarose from in front of the eyes and around the tail below the swim bladder so as to permit free eye and tail movements (referred to as a ‘head-mount’ or ‘head-fix’; for further explanation and representative images, refer to Bianco & Engert, 2015, and to Semmelhack et al., 2015). After embedding, larvae were allowed to recover overnight at 27.5°C prior to recording behavior. Behavioral experiments were then conducted at 7-8 *dpf* at room temperature. All larvae were recorded at 300 frames/s for ~4 minutes per trial, where each trial consisted of the high- to low-wavelength sequence of moving dots described above. We used a high-speed camera (PhotonFocus, Switzerland) to record eye and tail movements; during video recordings, larvae were illuminated from below with an infrared source. All other light sources were removed from the larvae’s vicinity during stimulus presentation. A period of >1 minute was allowed between each wavelength-respective dot stimulus to avoid visual adaptation.

Tail and eye digitization, trendline analysis, and quantification. We used custom Python scripts to quantify the position of the eyes and tail in the video recordings of individual trials. For eye movements, we defined vergence angle according to the parameters used by Bianco et al. (2011) as the difference between (initial and post-saccade) left and right eye position, where the nasal rotation of either eye causes an increase in vergence angle. Saccades were detected by convolving the vector describing the angular eye position of both left and right eye across the set of frames describing each trial, and summing the two trendlines. We then calculated the distribution of eye convergence angles for all trials for each larva independently, and used the first local minimum in the resulting distribution as the prey capture threshold. Distributions were usually bimodal, with the first peak representing the eyes in the unconverged ‘resting’ state, and the second, larger peak the converged state consistent with previously described prey capture paradigms (Mearns et al., 2019; Forster et al., 2020). For multimodal distributions containing three or more peaks, all peaks after the first were considered as converged ‘prey tracking’ ocular states. All ‘prey tracking’ and ‘non-prey tracking’ assignments were checked manually.

To analyze tail position for the duration of each prey capture trial, the tail was digitized and swim bouts extracted as described previously (Semmelhack et al., 2015). Briefly, tail position was quantified as a series of ~30-40 points covering the tail from its tip to the region at or just below the swim bladder, and the predicted midpoint location of the tail in each frame, as defined successively by the sequence of points in a given frame, added consecutively to produce a

positional curve describing the tail's movement across the duration of each ~4-minute trial. Tail movements were designated as 'prey tracking' only when the following conditions were satisfied: 1) they exhibited either the low amplitude/low strength, rapid oscillatory activity characteristic of forward swims or the sustained, unilateral bending motion of j-turns, and; 2) they occurred during a 'prey tracking' ocular state.

Behavioral data was organized in Excel and quantified using Prism (GraphPad). For each trial, prey tracking movements of the eyes and tail were counted individually for each of the color channels and used to generate four datapoints describing, respectively: 1) the number of prey capture eye vergences; 2) the ocular vergence angle of the largest magnitude nasal-directed shift in eye position; 3) the number of prey capture tail movements, and; 4) the summed duration of all prey capture tail movements. The set of four datapoints generated for each color channel were then compared to the same four sets from the other three channels, and presented as a series of dot plots displaying each of the above-described datasets and enumerating results for the complete set of four color channels. Note that no motion during a given color-channel trial is represented as a zero-magnitude datapoint.

6.3 Results

Zebrafish larvae exhibit hunting behavior in response to chromatic stimuli aligned with natural power distributions. To investigate patterns of motor output in response to prey-like visual cues, we used a head-fixed prey capture assay and video tracking system to monitor the frequency, duration, and amplitude of free eye and tail movements. 7-8 *dpf* larvae were tracked at 300 Hz under 960 nm illumination, and videos were processed by an automated behavioral classification system to extract and quantify activity during stimulus presentation. Moving dot stimuli were modeled on those described by Semmelhack and colleagues (2015), and presented as one of a UV, blue, green, or red spot traveling at a constant speed across a black background. Each complete trial, so defined, consisted of a randomly ordered sequence of UV, blue, green, and red dots, with each dot of a given color being played back-to-back three times across the viewing screen. Thus, for example, the first trial might have comprised a three-pass sequence of a moving green dot (sweep #1), followed by a three-pass sequence of one of UV, blue, or red (sweep #2), then another three-pass sequences of one of the two remaining colors (sweep #3), and finishing

with a three-pass sequences of the last color (sweep #4, after which the trial is complete) (**Fig. 1**). Note that, not infrequently within a single-color sweep, vergence angle for pass #2 and #3 responses would be relatively small compared to that for pass #1 due to their already being in a converged or semi-converged state.

Similar to the 2-photon *in vivo* setup detailed in chapters 3 and 5, the four LEDs used to recreate the zebrafish's four color channel visual system were spectrally aligned with the larval cone opsin absorption maxima (Zimmerman et al., 2018), and the relative power distribution set to match that of the daytime light environment of the species' native underwater hunting space (refer to chapter 4, **Fig. 1B**). This is a crucial step, since triggering behavior generally requires a higher activation threshold than does activating individual retinal cells or neural networks. Maximum contrast colors at any frequency along the zebrafish's tetrachromatic visual spectrum tend to evoke some response; but since our project is designed expressly to reproduce chromatic content and distribution in the teleost's natural environment—inquiring whether these parameters, rather than artificially-inflated ones, reliably trigger hunting routines—the chromatic stimuli in our experiment needed to match the relative photon catch rates at 365 nm, 411 nm, 467 nm, and 548 nm found in nature (Hunt et al., 2001; Chinen et al., 2003; Allison et al., 2004; Zimmerman et al., 2018). Only if, under these conditions, moving dot stimuli, and particularly those at the low wavelength-end of the spectrum, are sufficient to induce prey capture activities would this serve to further our hypothesis.

Indeed, for stimuli presented in all four color channels, the evoked eye and tail displacements included eye convergences, j-turns, and forward swims, all of which are distinct kinematic features of the hunting routine larvae display upon exposure to paramecia (McElligot & O'Malley, 2005; Bianco et al., 2011; Patterson et al., 2013). Nevertheless, it was immediately apparent that the number of responses per individual color sweep, as well as the strength and duration of each, differed in a short- versus long-wavelength-dependent manner. In a sample video recording of one trial, for instance, the UV dot elicited prey capture behavior in two out of three passes (**Fig. 2A**; eye movements only shown), and the Blue dot in three out of three (**Fig. 2B**), although eye movements in response to the second and third passes were low magnitude and might not have passed threshold to be included in quantification. Conversely, the Green dot elicited only one eye convergence (**Fig. 2C**), while the red dot, which during the recording period obtained a single

spontaneous eye convergence only after the stimulus had disappeared from the screen, elicited no quantifiable hunting behavior (**Fig. 2D**). Notably, while presentation of UV and blue dots tended to be accompanied by both eye and tail movements, responses to green and red dots often consisted of eye convergence only, with minimal or no tail motions of kind; and more typically, they produced no noticeable alteration in behavior of any kind.

It should be remarked that this is in line with our discussion of dual channel processing in chapter 5 (see section 5.4.2: ‘Color opponent computations in the AZ’), since long wavelength color circuits would be expected to be driven by other conspecific needs, and would in turn drive different motoric reflexes, than those for the UV guided prey capture channel. Within the limited scope of this experimental design, however, these results cannot substantiate that proposal to any degree; nor is it our purpose to do so.

Short wavelengths are better at eliciting prey capture than medium and long wavelengths.

To quantify behavioral responses to moving dots between the four color channels, we clustered the data representing eye and tail movements for each trial as a series of sixteen datasets, four each for UV, blue, green, and red. The following categories were used: 1) the number of prey capture eye vergences, which ranged from 0 to 2 for each sweep of the moving dot; 2) the ocular vergence angle of the largest magnitude nasal-directed shift in eye position, which was almost always the first; 3) the number of prey capture tail movements, including both forward swims and j-turns, and; 4) the summed duration of all prey capture tail movements. Final analysis was performed on those trials with eye vergence angles falling above the cut-off threshold (for fuller discussion, see this chapter’s Materials and methods, “Tail and eye digitization, trendline analysis, and quantification”).

Comparing the responses across the four color channels in each category revealed a number of interesting relations between short and long wavelength-evoked activity. First, eye convergences were more likely to occur in response to UV dots, followed by blue, green, and red ones, respectively (**Fig. 3A**). This trend was repeated in the ability of the four color dot to evoke high magnitude ocular vergences, with UV evoking the largest-angle convergences, green and red the smallest, and blue intermediate between the shortest and longest wavelength-stimuli (**Fig. 3B**). In a similar manner, but to a lesser extent, the duration over which the eyes remained in the converged

state also appeared to be associated with wavelength, although our short trial sequences precluded our being able to quantify this variable in any statistically verifiable way.

Differences among the capacity of individual wavelengths to induce tail movements followed the same general pattern (**Fig. 3C-D**), with both frequency and duration of hunting motions most pronounced in the UV and blue channels, respectively. By contrast, green and red often elicited no tail movements of any kind, and even when they did exhibit motions which could be considered as either j-turns or forward swims, these were consistently of shorter duration (**Fig. 3C**) and smaller amplitude (data not shown) than for the two lower-wavelength channels. The association between the stimulus color's potential for eliciting large-scale ocular vergences as well as proportionately strong and numerous tail movements can be explained by the demonstrated correspondence of eye and tail motions in response to prey-like objects (Semmelhack et al., 2015).

6.4 Discussion: Connecting RGC responses and behavior

Previous research has suggested that zooplankton scatter light in the 320-390 nm range (Novales Flaminique, 2012, 2016; Zimmerman et al., 2018; Yoshimatsu et al., 2019; reviewed in Losey et al., 1998; see also chapter 1, section 1.3: 'the zebrafish as a model of visual system form, function, and adaptation', pp.). To trichromats such as macaques and humans, equipped with the primates' distinctive 430-530-561 nm/blue-green-red spectral sensitivities (Schnapf et al., 1988), these aquatic microorganisms are largely transparent when viewed against a background illumination (Johnsen & Widder, 2001). But to zebrafish, whose tetrachromatic cone complement enables comparatively short-wavelength vision, they are thought to appear as UV-bright spots silhouetted against the sunlit upper water column (Novales Flaminique, 2012, 2016; Zimmerman et al., 2018). This idea has recently found confirmation in a study which used specially designed camera-filter systems to film free-swimming paramecia in the larval zebrafish's UV opsin channel and, separately, the green/red one (Yoshimatsu et al., 2019; for fuller discussion, see chapter 5, section 5.4: 'A diversity of long and short wavelength biased ON circuits to match the visual statistics of a freshwater environment', pp.). The particular appeal of the presumptive 'natural' appearance of the larvae's prey-object-of-choice is also corroborated by the hunting routines elicited in laboratory settings by both live paramecia (Hernandez et al., 2002; McElligott & O'Malley, 2005; Patterson et al., 2013; Trivedi & Bollman, 2013; Semmelhack et al., 2015; Marquez et al., 2018;

Mearns et al., 2019) and, more indirectly, high-contrast spots of light (Bianco et al., 2011; Bianco & Engert, 2015; Semmelhack et al., 2015; Antinucci et al., 2019).

In the present chapter, we have examined the proposed link between the zebrafish's inner retinal circuitry and specific chromatic features of visually guided hunting behaviors. We have shown that shorter wavelengths, extending from the UV and into the blue, are capable of provoking frequent, long-duration, high magnitude eye and tail movements similar to those displayed by free-swimming larvae while tracking prey (McElligott & O'Malley, 2005; Patterson et al., 2013). This response is most pronounced at the 365 nm range, and degrades progressively towards the infrared end of the visible spectrum, in line with our previous analysis of the number and distribution of color sensitive ON circuits among RGCs (see chapter 5) as well as those of BCs (Zimmerman et al., 2018) and PRs (Yoshimatsu et al., 2019). Furthermore, we have shown that this trend is preserved even when the relative power distribution between the color channels is scaled to mimic the zebrafish's natural white. Thus, even when the relative photon catch for UV is a decreasing fraction of that for blue, green, and red, respectively, the low wavelength/low power stimuli nevertheless elicit hunting movements of greater magnitude, and with greater consistency, than those presented at higher wavelengths and relatively increased power.

Altogether, our results provide the first direct behavioral evidence for the notion, heretofore supported primarily by anatomical and physiological data, that the larval zebrafish retina is specialized for detecting UV-biased features of prey-like objects in order to initiate prey capture. Moreover, it is designed to do so at the relatively low signal power and signal-to-noise ratio which characterize short wavelength visual cues in the species' native underwater environs. Although due to their restricted methodological scope and inability to distinguish between retinal regions or cells, these data and conclusions should be considered preliminary and incomplete, they do nevertheless broadly confirm the microevolution-based hypothesis put forward in the preceding chapters. Equally important, they offer a number of promising jumping-off points for subsequent investigations seeking to consolidate and build upon this proposed link.

Prospects for future investigation. In the experiments for this chapter, we did not perform calcium imaging, photoablation, or photoactivation and tracing, which could have solidified the connection, at present only a correlation, between the anatomy and physiology and the production of response patterns by environmental stimuli. With this in mind, as well as the relatively limited

scope of the methods we did employ, our data and conclusions leave open quite a few intriguing experimental opportunities.

In the first place, our method did not distinguish which retinal quadrants were predominantly responsible for the UV-based perception of prey objects. Although physiological recordings taken from the inner (see chapter 5) and outer retinal layers (Zimmerman et al., 2018; Yoshimatsu et al., 2019) implicates the ventrotemporal area, and most significantly the AZ, for this role, establishing this with certainty would require presenting our stimulus paradigm individually to the ocular quadrants by systematically ablating or otherwise occluding cells in the remaining three quadrants. Ideally, such an experiment would show that the AZ is necessary and sufficient for prey detection and the commencement of hunting. However, even demonstrating smaller, but statistically significant contributions from the ventral, dorsal, and nasal quadrants, measured as partial reductions in eye, tail, or jaw movements, would have highlighted the importance of the *area temporalis* in prey hunting.

Likewise, neither did we attempt to differentiate which RGC types detected the colors and contrasts which, along with size, shape, and speed, collectively parameterize prey-like stimuli. Here again, the most efficient, tried-and-true means of accomplishing this would be targeted genetic ablation of type-specific marker-driven *UAS:dendra*— for example, the B2 and D1 morphologies described by Semmelhack and colleagues in their 2015 study. By systematically killing one genetically distinguishable type at a time, and then all those estimated responsible for the AZ's exclusive physiological character, it would be possible to ascertain the individual functional groups responsible for encoding different features of the prey response. Of course, for most of the past two decades, this has been a difficult prospect for researchers of zebrafish vision and visual circuitry; despite the teleost's remarkable genetic accessibility, only a few, and unspecific, markers for RGCs were known, none of which targeted fewer than several distinct types (Tokumoto et al., 1995; Kay et al., 2005; Xiao et al., 2005). Such an approach has only recently become feasible by the identification of RGC type-specific markers using single-cell transcriptomics (Kölsch et al., 2021).

The >230 RGC types which have now been classified enable as well other methods for systematically studying the architectural and physiological profile of the zebrafish inner retina. With regard to the AZ and its theorized association with prey-object perception, one knowledge

gap which stands out in particular is the morphological and axonal projections of cells responsible for detecting and initiating motor routines to prey-like objects. Linking individual neurons to network function and behavior would likely be a complex and ticklish behavior, requiring the use of multiple transgenes and a combination of anatomical and functional fluorescent reporters. Although there are a number of market options available for consideration when designing such an experiment, one possibility would be the simultaneous driving of a nuclear-localized calcium or voltage indicator and a photoactivatable GFP or RFP localizing to the cytoplasm. (The most promising such method is the FuGIMA technique, described in Forster et al., 2017; Forster et al., 2018; see also Del Maschio et al., 2017). In this scenario, nls-GCaMP/Voltron expressed at a constitutive but low level among the targeted population of RGCs would be monitored by 2-photon microscopy for calcium/ion transients in response to swimming paramecia or virtual prey stimuli. Although larval RGCs are very densely packed, nls-indicators do permit distinguishing between individual somata, since the empty cytoplasmic space provides an $\sim 0.5\text{-}1.0\mu\text{m}$ ring around the color-marked nuclear compartment; those somata that exhibit strong transients to prey-like objects could then be photoactivated and the diffusible fluorescent protein traced along the extent of the cell's neurites. In this manner, the full anatomical profile of individual prey-responsive RGCs could be characterized similar to the projectome mapping done by Robles and colleagues (2015), but with a greater degree of accuracy, efficiency, and in sufficient population size to permit statistical relations to be assessed between dendritic structures and the tectal, pretectal, or other axonal targets of fluorescing cells, as well as between their architectural characteristics and their physiology and behavioral associations.

It is of practical consequence that although a non-selective RGC marker such as *Ath5* or *Islet2b* would be serviceable for such an investigation, coupling a FuGIMA sequence to a range of single type-selective promoters which together target the full complement of RGCs would permit focusing on small subsets of the population at a time. The fluorescent signal would then be more sparse, helping to preclude signal contamination making individual RGC somata less easily distinguishable during functional imaging, as well as incidental photoactivation of surrounding cellular compartments.

The prospective projects outlined above are only three of the numerous exciting experimental possibilities which propose themselves with regard to the AZ's influence on emergent behavior.

Other questions which remain to be addressed include the distribution of prey-responsive RGC types across the retinal hemisphere, their upstream connectivity to BC and AC types, interneuron connectivity with RGC terminals in the AFs and optic tectum, and the extent to which the blue channel contributes to the ability of AZ RGCs to detect prey. Elucidating each of these aspects of how inner retinal structures guide visual prey capture will be an important goal for vision researchers.

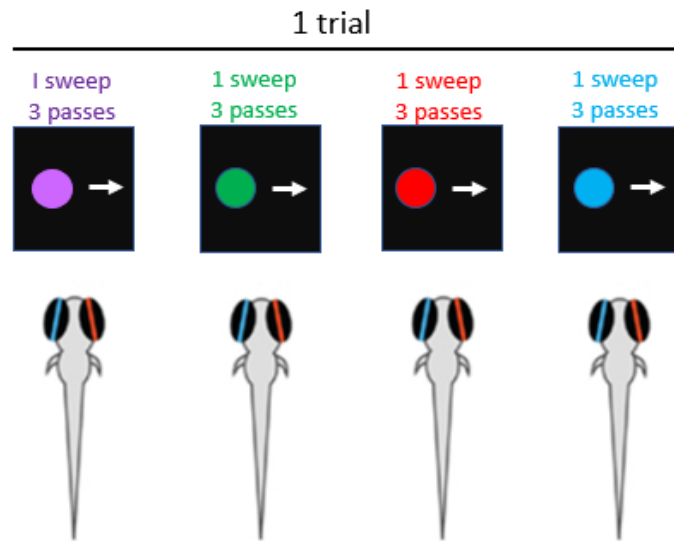


Figure 1: Stimulus battery and trial sequence for behavioral recording.

Sample stimulus paradigm for behavioral testing of hunting responses to individual chromatic 'moving dots'. In this sequence, a UV dot is played first, appearing on the left-hand side of the screen and exiting right for a total of three 'passes' in the complete UV sweep. Green, red, and blue sweeps, respectively, follow in the same manner, completing the twelve passes constituting one trial.

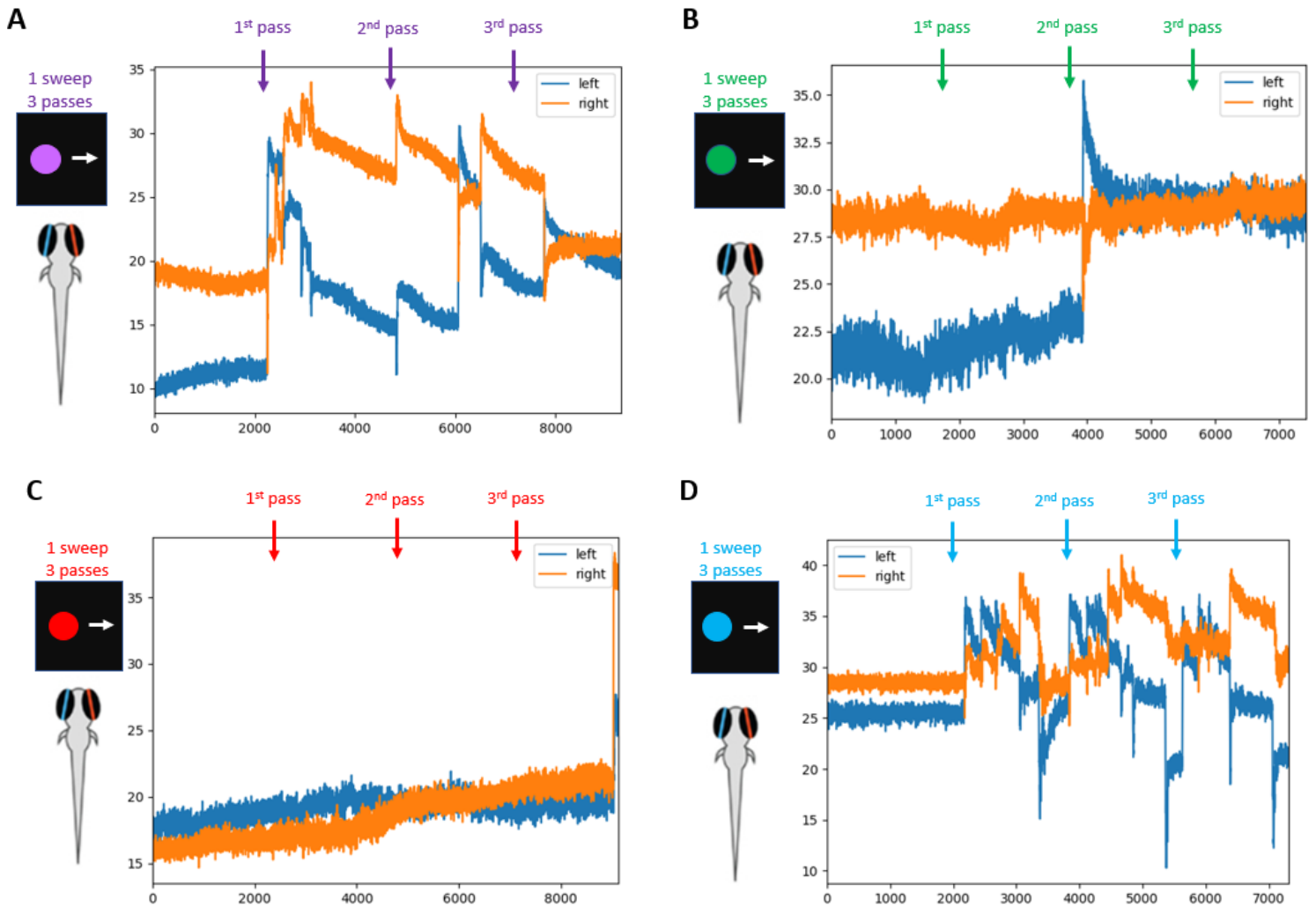


Figure 2: Sample recording from head-fixed larva in response to moving dots in four colors.

(A-D) Responses to UV (A), Green (B), Red (C), and Blue sweeps (D) which constitute one trial. Note that in (A-B), the eye movements following the onset of the third color sweep, in the case of the UV dot (A), and of the second color sweep, in the case of the Green dot (B), would not be counted as prey-capture converges since the eyes are diverging. Neither would the eye movement depicted in (C), since the movement, although a convergence, occurred after the Red dot had disappeared off the right side of the screen.

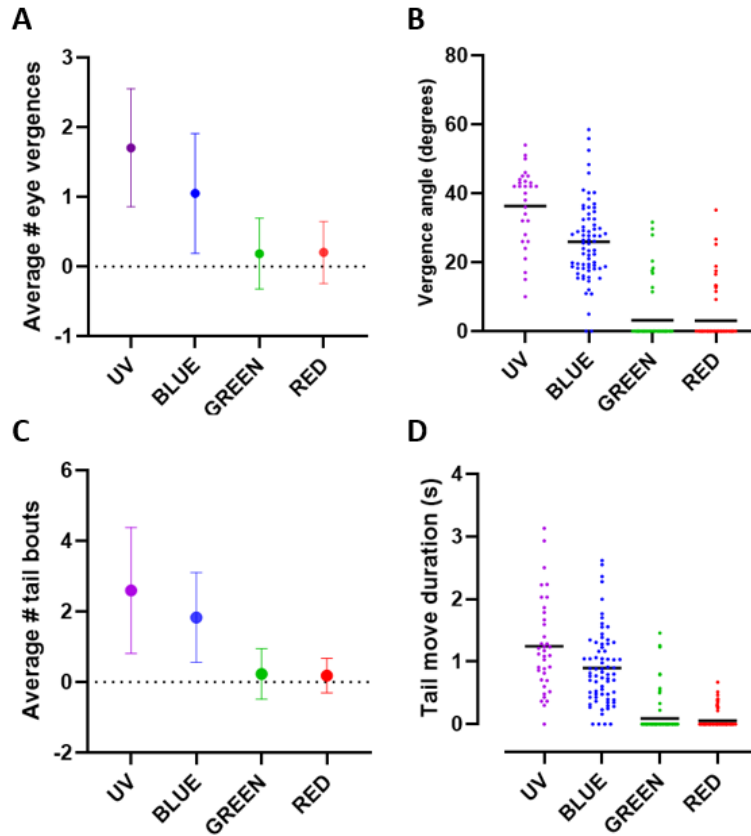


Figure 3: Chromatic preferences in prey capture stimulus-response paradigms.

(A) Mean number of ocular vergences per color channel per trial. For example, when presented with a UV dot sweep, the larva might respond by converging its eyes in response to each of the three passes, quantified as 3/3; when presented with a Green dot sweep, it might respond with vergence to only one out of the three passes, quantified as 1/3; and the same for Red and Blue. Dot and lines represent mean and standard deviation, respectively. Results of comparison by one-way ANOVA between: UV and B ($P=0.0008$, extremely significant, ***), UV and G ($P<0.0001$, extremely significant, ****), and UV and R ($P<0.0001$, extremely significant, ****). **(B)** Average ocular vergence angle per pass in which the larva responds to at least one pass in any of the four color channel sweeps. Black line represents mean. Results of comparison by one-way ANOVA between: UV and B ($P=0.0001$, extremely significant, ***), UV and G ($P<0.0001$, extremely significant, ****), and UV and R ($P<0.0001$, extremely significant, ****). **(C)** Average number of tail bouts per color channel per trial. For example, when presented with a UV dot sweep, the larva might respond with a J-turn and a forward swim to the three passes in that color channel, quantified as ‘2’ prey hunting movements; when presented with a Green dot sweep, it might not respond with any hunting movements, quantified as ‘0’; and when presented with a Red dot, there might be no tail motion at all, which would similarly be quantified as ‘0’. Dot and lines represent mean and standard deviation, respectively. Results of comparison by one-way ANOVA between: UV and B ($P=0.0013$, very significant, **), UV and G ($P<0.0001$, extremely significant, ****), and UV and R ($P<0.0001$, extremely significant, ****). **(D)** Average duration of tail bouts per color channel per trial. For example, when presented with a UV dot sweep, the larva might respond by performing a J-turn for 1 second followed by a 0.5 second forward swim, in which case the total value (duration) for that individual point will be 1.5 seconds. Black line represents mean. Results of comparison by one-way ANOVA between: UV and B ($P=0.0005$, extremely significant, ***), UV and G ($P<0.0001$, extremely significant, ****), and UV and R ($P<0.0001$, extremely significant, ****).

7. CONCLUDING DISCUSSION

7.1 Decoding the retina

In the case of the tetrachromatic zebrafish retina, the efficient coding hypothesis (Attneave, 1954; Barlow, 1961; Simoncelli & Olshausen, 2001) predicts an investment of retinal hardware according to the demands of an anisotropic visual space, with long and short wavelength sensitivities and processing capacities differentially organized according to the relevant features present in the different areas of the species' native visual environment. The reorganizations at both the anatomical and functional level known to be present in the outer retinal layers of the *area temporalis* (Schmitt & Dowling, 1999; Zimmerman et al., 2018; Yoshimatsu et al., 2019), and the success with which they have been connected to experimental paradigms of visually-evoked prey capture (Bianco et al., 2011; Semmelhack et al., 2015; Mearns et al., 2019; Yoshimatsu et al., 2019), lend credence to the idea that adaptations toward efficiency operate in the outer layers, at least, of the larval zebrafish retina.

With regard to those retinal layers which are relatively more thoroughly-characterized in terms of form, physiology, and predictive behavioral function, we have already seen how pronounced are structural anisotropies within the 7 *dpf* larval retina, which extend right down to the molecular-cellular level. Within the photoreceptor layer, in addition to the density differentials of rods and cones (Schmitt & Dowling, 1999; Yoshimatsu et al., 2019), the PRs themselves possess a number of features which vary not only between types but among cells of the same type positioned in different regions. For example, the size of UV cone outer segments and ellipsoid bodies varies across the eye: within the acute zone, they are enlarged in length by as much as an order of magnitude compared to those in other regions, increasing their load-bearing capacity for vital functional and metabolic machinery, including the amount of phototransduction cascade machinery each cell can contain, its mitochondrial content, and its calcium-bearing capacity, as well as boosting its photon-catch efficiency (Yoshimatsu et al., 2019). These region-specific structural variations extend as well into the second stage of visual processing, with both type-specific BC immunoreactivity and the density, shapes, and sizes of BC terminals changing along with eye position (Zimmerman et al., 2018). Furthermore, these anatomical features of PRs and BCs have been shown to manifest as region-specific differences in their network function, with respect to both intracellular and intercellular signaling mechanisms (Zimmerman et al., 2018;

Yoshimatsu et al., 2019). Finally, that such region-specific outer retinal profiles have been matched with creditable success to behaviorally relevant visual cues in experimental paradigms (Semmelhack et al., 2015; Mearns et al., 2019; Yoshimatsu et al., 2019) provide strong confirmation that the photoreceptor, outer nuclear, and outer plexiform layers function in accordance with a minimal code for sensory transmission.

Nevertheless, the crucial step from retinal physiology to brain areas capable of evoking motor behavior rests with the neuronal population— the RGCs— which completes the retinal circuit, and which is ultimately responsible both for the final integration of its light signals, prey capture-related or otherwise, and the innervation of retinorecipient midbrain structures projecting to the relevant motor-output areas. Only by functionally linking the outer retina’s putative prey capture circuit to brain regions which mediate the perception of and response to prey (Semmelhack et al., 2015; Antinucci et al., 2019) can the conjunction of the two networks be concluded with confidence. This thesis, along with the accompanying publication (Mingyi, Bear, et al., 2020), constitute the first comprehensive attempt to bridge the optic chiasm between eye and brain, or more strictly speaking, between the eye and certain basic patterns of motor output.

In investigating the role of RGCs in the retina-to-brain circuit, our project comprised a threefold objective. Our first aim has been to characterize the RGC anatomy and physiology in light of the efficient coding hypothesis and, more specifically, with reference to the region-specific sensory integration capacity identified in outer retinal layers. As an important corollary of this primary aim, our behavioral data provides grounds, albeit preliminary and speculative, for assuming that cells in all retinal layers— and so necessarily RGCs— are capable of responding reliably and robustly to stimuli whose component features are derived from the statistics of natural settings, as they would need to be for us to draw conclusions about their structural and functional profile which are behaviorally significant. As a second, no less consequential objective, we have sought throughout the discussion to systematically identify questions which remain partially or wholly unanswered by our study, and to outline possible research methods toward achieving a complete account of one of the most important models of vision research, both in terms of theoretical study and translational research. Last and— some would say— least, we endeavored to contextualize our experimental observations within an anachronistic, but still germane, historical debate about the

meaning which our theories bring to the data. Since this is what we started with, it seems fitting to make concluding remarks as to the first two objectives with respect to the third.

7.2 Paley and Darwin again: paradigms within which facts are defined, highlighting the notion that a single idea can still result in a conflict of ideas

David Hume's wonderfully expressive, characteristically pithy, and assuredly ironical articulation of the philosophical question underlying a great deal of Enlightenment and post-Enlightenment scientific study will serve as point of departure for this discussion, since indeed he is the immediate intellectual precursor of William Paley and Charles Darwin, and the figure whom both were to some extent addressing (Huntley, 1972; McLean, 2019). Hume bids us to "consider, anatomize the eye; survey its structure and contrivance; and tell me, from your own feeling, if the idea of a Contriver does not immediately flow in upon you with a force like that of sensation?" (Hume, 1776, 56).

Having considered and anatomized, we are in a position to respond at least to the explicit meaning of the question; that contrivance, whether in the theist's sense of intentional and intelligent design, or that of the methodological naturalist's interplay of physical and biological forces, is indeed a fitting vignette in the case of our model of visual form and function. The data presented in this thesis and the conclusions which seem most reasonably drawn from them demonstrate a remarkable fidelity between the zebrafish larva's AZ ganglion cells and the environmental and behavioral requirements imposed upon their physiology by the teleost's native habitat. Thus too, they accord favorably with the expectations of fine-tuning in the 19th century sense, with those of efficient coding in the 20th century use of the term (Simoncelli & Olshausen, 2001), and more particularly with regard to the zebrafish, with the premises drawn from recent research in the field of visual ecology (Schmitt & Dowling, 1999; Robles et al., 2014; Semmelhack et al., 2015; Zimmerman et al., 2018; Yoshimatsu et al., 2019).

In summary, the pronounced reorganizations we have here detailed within the inner retina encompass three broadly-defined levels of experimental inquiry:

1. the molecular and cellular level, including organelle relocation, anisotropic neuronal-type and type distributions, and comparative morphological classification between nasal and AZ;
2. the circuit level, including a functional profile of AZ RGCs, how this profile differs from that of other retinal regions, and a discussion of multi-channel signaling as a possible explanation for the unique but superimposed spectral, spatial, and temporal characteristics of AZ RGCs;
3. the level of chromatic perception as behavioral output, though to a lesser degree than the preceding, including motor responses to prey-like stimuli which mimic naturalistic color contrasts.

In working at these three levels of experiment and analysis, we have been guided by the expectation that environment, behavioral requirements, and the computational capacities and limitations of retinal neurons ought to be investigated in combination in order to comprehensively and comprehensibly elucidate the function of neurons and neural systems within the AZ and prey-hunting-related activity. In line with this expectation and our original hypothesis, we have shown that the number, morphological distribution, and functional profile of RGCs varies across the eye, that these regional variations are most pronounced within the AZ, and that the AZ specialization may be linked to particular hunting behaviors insofar as prey-like stimuli are matched to the tuning preferences of retinal cells in general. In addition, our somewhat eclectic blend of anatomical, physiological, behavioral, and computational tools demonstrates the power of combining techniques for the purpose of gaining a thorough understanding of the larval AZ, and of the zebrafish retina as a whole, as well as the visual systems of other model organisms. (For similar methodological approaches which inspired this project, see Semmelhack et al., 2015; Baden et al., 2016; Zimmerman et al., 2018; Yoshimatsu et al., 2019). We conclude that the inner AZ contains a population of UV-responsive, ON-sustained RGCs, largely unique to this region of the retina, which is likely linked to their behavioral requirement to detect and pursue UV-bright zooplankton (**Fig. 1**). And we admit, ‘from our own feeling’, and putting aside the idea of a Contriver, that some astonished inkling of contrivance does indeed force itself upon us.

...But what would Darwin and Paley have to say to all this, and to one another once having read it? Quite frankly, it would probably be nothing we haven’t heard before reading either of their

texts. At a guess, the naturalist would stick to his naturalistic guns, the theologian to his theological ones. The first would thank chance, necessity, and pressure exerted by various environmental factors, the other would thank God, grace, and providence, and both might append a footnote (if we were lucky) to their pages mentioning that, indeed, the intricate and multifaceted design of a certain teleost's eye adds a grain of sand to the scale in favor of their theory. In other words, each would assume himself justified from the perspective of his paradigm, and would answer Hume in the affirmative.

But then, what do Paley and Darwin see in Hume's question, or rather his challenge, which we do not? What do we read, or fail to read, which they understand differently by such basic questions as— what have we discovered? what can we say about it? and what remains to discover and to say?

7.3 Not as easy as shooting fish in a barrel: the antinomy of pure empiricism

Another preeminent figure of philosophical and scientific thought would likely also have given his averment to Hume's enquiry, and would likewise have done so from the standpoint of a third perspective, for reasons entirely his own and entirely distinct from those of the two figures whose debate introduced our essay. Emmanuel Kant was one of the first Enlightenment thinkers to point out how science, too, must as a practical matter necessarily employ the idea of teleology— of exploring means and methods in relation to perceived or hypothesized ends— although requiring it to do so in a manner detached from theological consideration (Kant, 1790, 245-258). Thus, he would have amended his 'yes' with the following clarification, insofar as 'clarification' is the proper term for any passage in the complex train of reasoning which constitutes his *Critiques*:

When we say, for example, that the crystalline lens in the eye has the purpose of focusing, through a secondary refraction, the light rays emanating from a certain point into a point on the retina, we are merely saying that we think the representation of a purpose in the causal action of nature in producing the eye, [and that we think it] because such an idea serves as a principle for conducting our investigation concerning this part of the eye, and thus also assists us to devise possible means of enhancing the relevant effect. But this does not yet involve attributing to nature a causality in accordance with a representation of ends, that is, an intentional action (343).

Kant's statement puts in contrast the need which both Darwin and Paley have for assuming a means-end relation in their research, the former for pragmatic reasons (Darwin, 1860), the latter for philosophical ones. And it reminds us that we, too, must make similar judgments in pursuing scientific hypotheses such as those defended in this thesis; but also that in so doing we have an obligation to be self-consciously cautious as to the supra-empirical conclusions we draw from our data in light of those judgments, at least within the context of the study itself.

In summing up the results of our investigation and laying out the conclusions framed by our prey-capture hypothesis, this would no doubt have been the place to quote from either of our two original contending parties, and by so quoting to establish by implication whether we were also replying to the implicit meaning of Hume's remark on the classic eye-evolution debate in terms of Paley's theological naturalism or Darwin's methodological one— flagrantly disregarding Kant's admonishment all the while. But in light of this paper's technical- and research-based focus, such a predictable either-or dichotomy, while not necessarily yielding allegiance wholesale to one or the other faction, would be in some sense to translate into modern terms a dispute which was necessarily not framed that way. It would also be to weigh in on what is also, essentially, an interminable question (Kant, 1791, 340-344) in that the basic presuppositions and background beliefs from which both methodological naturalist and theological naturalist reason are incommensurable (for an excellent reference on competing rationalities, read MacIntyre, 1988, 1-11 and 326-348). As implied by Hume's multilayered question, such a dispute, unresolved, likely irresolvable, and which only on the surface appears solidly and straightforwardly empirical, has philosophical roots which long preceded both of the 19th century disputants who most clearly delineated two different approaches to answering a single particular question— approaches to a question which emerged in the 20th century in the academic study of visual ecology.

Let us, then, rather admit with Kant all we can say on the matter, which is all we can say on most matters of real significance, even when our process of reasoning *is* based on shared premises:

The concept of natural ends is therefore merely a concept of the reflective power of judgment for the sake of exploring the causal connection in objects of experience. Employing a teleological principle for the explanation of the inner possibility of certain natural form leaves it undecided whether their purposiveness is intentional or unintentional. A judgment asserting either one or the other conclusion would be a determining ['it is

known or knowable'– J.B.] rather than a reflective ['it is possible, but not known or knowable'– J.B.] one, and the concept of an end of nature would then no longer be a mere concept of the power of judgment, for immanent (empirical) employment, but would be bound up with a concept of reason, of an intentionally acting cause set over nature, irrespective of whether we wished to an affirmative or negative judgment in this case (Kant, 1791, 343-344).

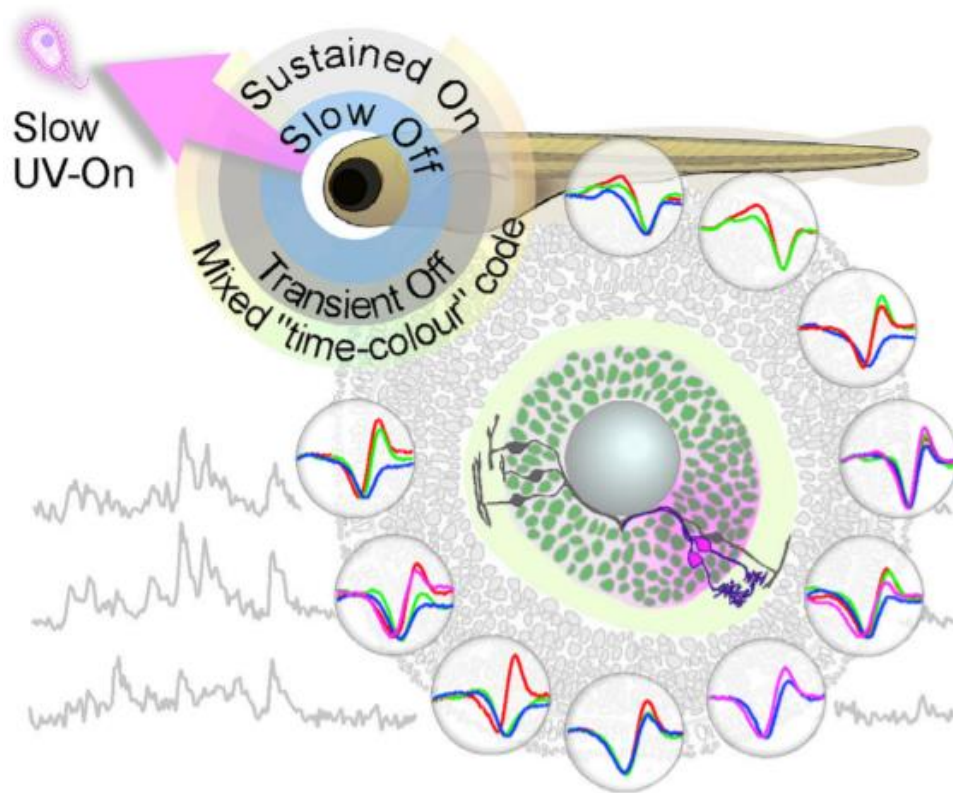


Figure 1: Model for acute zone RGC structure and function in comparison to the retinal whole.

While a variety of long- and short-wavelength chromatic stimuli elicit responses from RGCs positioned in the ventral, dorsal, nasal (Zhou, Beat, et al., 2020), those type-specific populations within the ventrotemporal AZ exhibit a UV dominance in response to spectrally naturalistic full-field stimuli.

8. LITERATURE CITED

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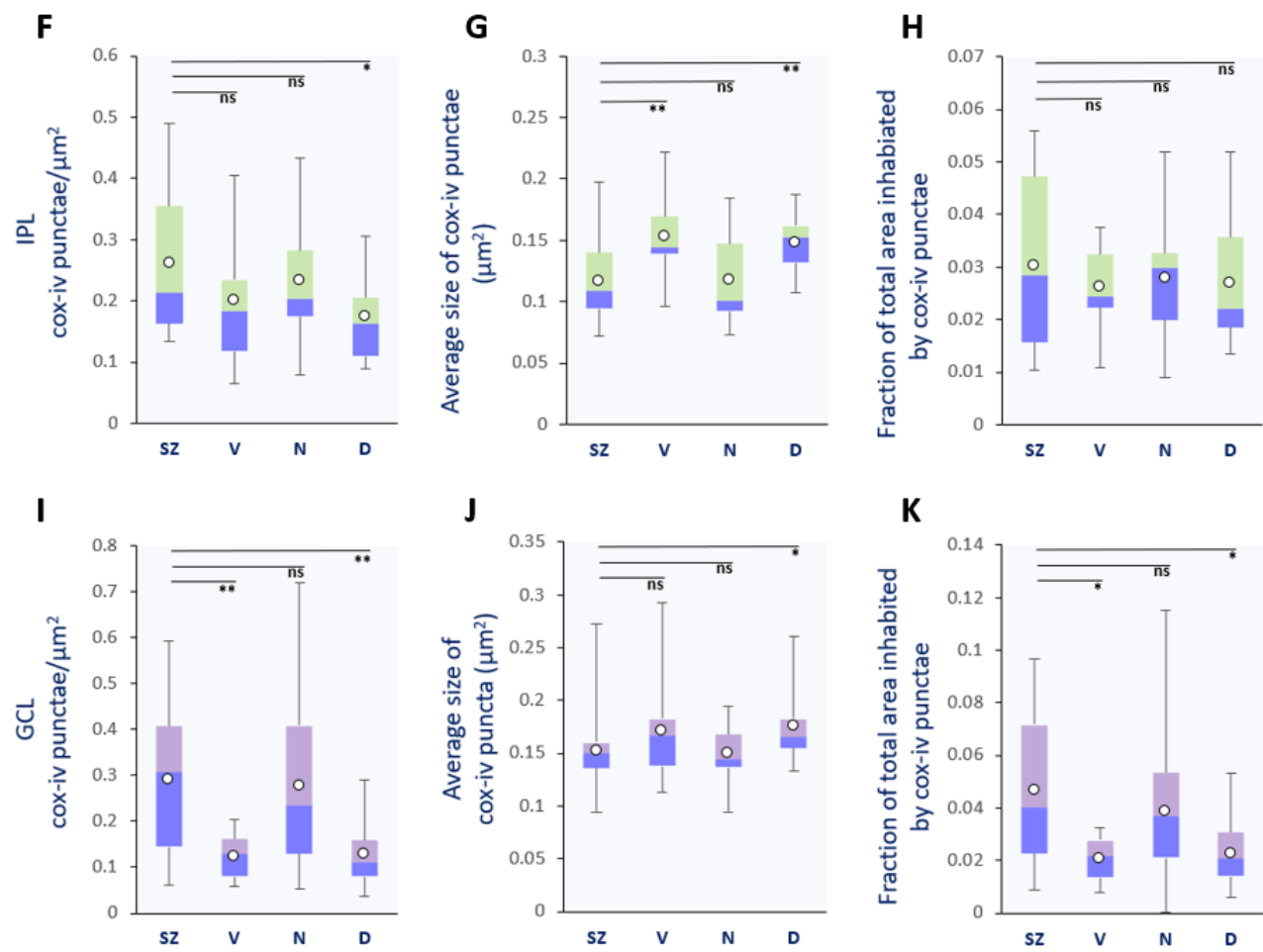
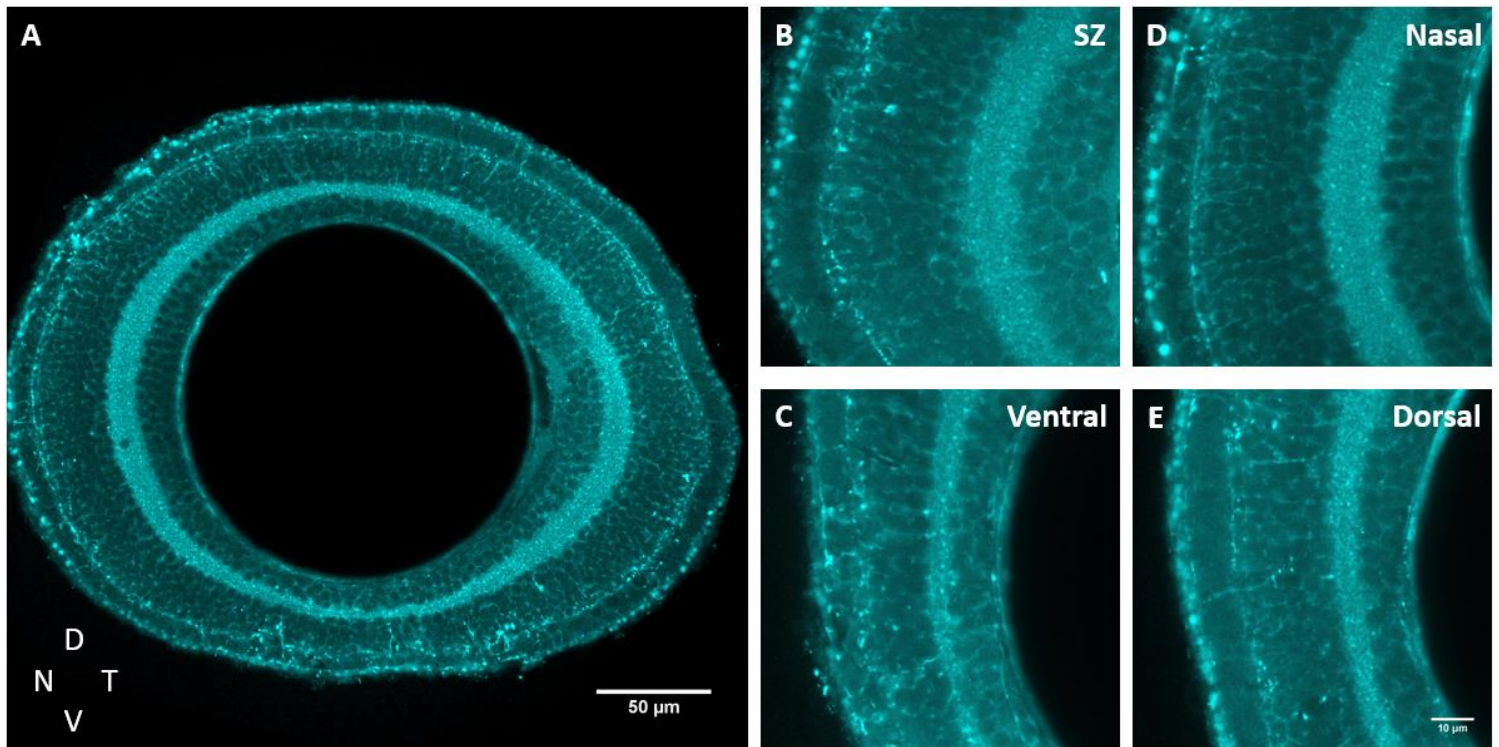
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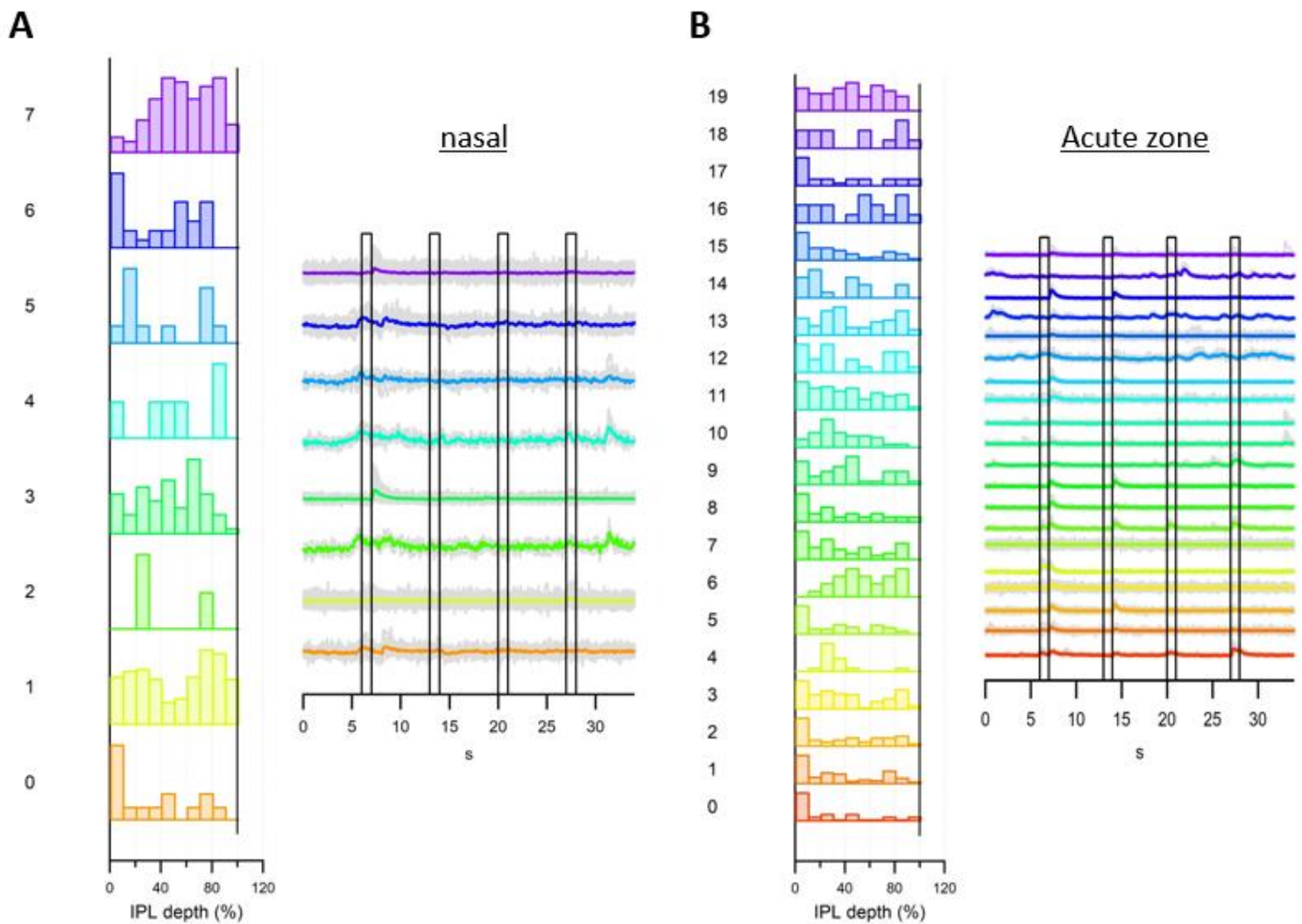
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9. Supplementary figures



Supplementary Figure 1 | related to chapter 2: Region-specific characteristics of cox-iv punctae within the inner retina.

(A) Sagittal section across 7 *dpf* larval retina, stained for Cox-iv, a mitochondrion-specific antigen. **(B-E)** Enlarged sections from **(A)** showcasing the different allocations of mitochondria within SZ **(B)**, ventral **(C)**, nasal **(D)**, and dorsal **(E)**. **(F)** Comparison of the number of cox-iv punctae per square micrometer in the IPL between SZ and V ($P=0.1745$ (not significant), Mann-Whitney $U=89$, $n_{SZ}=21$ ROIs, $n_V=12$ ROIs), SZ and N ($P=0.7674$ (not significant), Mann-Whitney $U=178$, $n_{SZ}=21$ ROIs, $n_N=18$ ROIs), and SZ and D ($P=0.0352$ (significant, *), Mann-Whitney $U=77$, $n_{SZ}=21$ ROIs, $n_D=13$ ROIs). Points indicate averages (SZ=0.261 puncta/ μm^2 , V=0.0201 puncta/ μm^2 , N=0.232 puncta/ μm^2 , D=0.175 puncta/ μm^2). **(G)** Comparison of average puncta size in the IPL between SZ and V ($P=0.0061$ (very significant, **), Mann-Whitney $U=54$, $n_{SZ}=21$ ROIs, $n_V=20$ ROIs), SZ and N ($P=0.7842$ (not significant), Mann-Whitney $U=199$, $n_{SZ}=21$ ROIs, $n_N=20$ ROIs), and SZ and D ($P=0.0018$ (very significant, **), Mann-Whitney $U=605.5$, $n_{SZ}=21$ ROIs, $n_D=12$ ROIs). Points indicate averages (SZ=0.117 μm^2 , V=0.153 μm^2 , N=0.117 μm^2 , D=0.151 μm^2). **(H)** Comparison of the fraction of total area inhabited by puncta in the IPL between SZ and V ($P=0.6961$ (not significant), Mann-Whitney $U=105$, $n_{SZ}=21$ ROIs, $n_V=11$ ROIs), SZ and N ($P=0.8991$ (not significant), Mann-Whitney $U=184$, $n_{SZ}=21$ ROIs, $n_N=18$ ROIs), and SZ and D ($P>0.9999$ (not significant), Mann-Whitney $U=126$, $n_{SZ}=21$ ROIs, $n_D=12$ ROIs). Points indicate averages (SZ=0.0302, V=0.0262, N=0.0289, D=0.0279). **(I)** Comparison of the number of cox-iv punctae per square micrometer in the GCL between SZ and V ($P=0.0047$ (very significant, **), Mann-Whitney $U=43$, $n_{SZ}=20$ ROIs, $n_V=11$ ROIs), SZ and N ($P=0.7674$ (not significant), Mann-Whitney $U=178$, $n_{SZ}=21$ ROIs, $n_N=18$ ROIs), and SZ and D ($P=0.0352$ (very significant, **), Mann-Whitney $U=77$, $n_{SZ}=20$ ROIs, $n_D=14$ ROIs). Points indicate averages (SZ=0.289 puncta/ μm^2 , V=0.0124 puncta/ μm^2 , N=0.299 puncta/ μm^2 , D=0.129 puncta/ μm^2). **(J)** Comparison of average puncta size in the GCL between SZ and V ($P=0.2273$ (not significant), Mann-Whitney $U=80$, $n_{SZ}=20$ ROIs, $n_V=11$ ROIs), SZ and N ($P=0.6360$ (not significant), Mann-Whitney $U=182$, $n_{SZ}=20$ ROIs, $n_N=20$ ROIs), and SZ and D ($P=0.0225$ (significant, *), Mann-Whitney $U=75$, $n_{SZ}=21$ ROIs, $n_D=14$ ROIs). Points indicate averages (SZ=0.152 μm^2 , V=0.171 μm^2 , N=0.151 μm^2 , D=0.176 μm^2). **(K)** Comparison of the fraction of total area inhabited by puncta in the GCL between SZ and V ($P=0.0108$ (significant, *), Mann-Whitney $U=49$, $n_{SZ}=20$ ROIs, $n_V=11$ ROIs), SZ and N ($P=0.7353$ (not significant), Mann-Whitney $U=187$, $n_{SZ}=20$ ROIs, $n_N=20$ ROIs), and SZ and D ($P=0.0150$ (significant, *), Mann-Whitney $U=71$, $n_{SZ}=21$ ROIs, $n_D=12$ ROIs). Points indicate mean (SZ=0.0466, V=0.0207, N=0.0425, D=0.0227).



Supplementary Figure 2: Responses of RGC dendrites and somata to UV step stimulus.

(A-B) RGC responses to UV step stimulus in the nasal (A) and AZ (B). Clustering is by k-means, and the responses of somata and dendrites are not distinguished. Black boxes represent onset and duration of each UV ON stimulus. Note that the AZ constitutes a more variegated response profile than in the nasal, and that each AZ RGC functional type response appears to stratify across a greater extent of the IPL depth.

