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Zebrafish Colour Vision:

a survey of spectral processing in the zebrafish
visual system.

Philipp Bartel

Submitted for the degree of Doctor of Philosophy

University of Sussex

September, 2020

Declaration

I hereby declare:

The thesis entitled: “Zebrafish Colour Vision” submitted for the degree of Doctor of Philosophy presents only my own work. Collaborative work and external data are explicitly indicated throughout the manuscript.

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another university for the award of any other degree.

Philipp Bartel

Chapter 2: Light Synthesiser. This is a technical note that presents my own work.

Chapter 3: Cones. This research has been published in *Science Advances* 7(24). In brief:

TY, PBa and TB designed the study, with input from CS, FKJ and PBe; TY generated novel lines and performed 2-photon data collection and pre-processing; TY also performed anatomical imaging, tracing and data analysis, with input from CS. PBa built the light-stimulator with input from FKJ. PBa also performed natural imaging data analysis, with input from TB and PBe. CS built the computational HC-cone model with input from PBe, and performed clustering analyses. TY, PBa, and CS performed statistical analyses. TB wrote the manuscript with inputs from all authors.

Chapters 4: Bipolar Cell Terminals. This research has been published in *Current Biology* 31. Conceptualization, PB, TY, TB; Methodology, PB, TY, FKJ; Investigation, PB, TY; Data Curation, PB, TB; Writing – Original Draft, TB; Writing – Review & Editing, TB, TY, FKJ; Visualization, TB, PB; Supervision, TB; Project Administration, TB; Funding Acquisition, TB.

Chapter 5: Brain Data. The first portion of the chapter shows interim work by myself exclusively. The latter portion has been published in *Current Biology* 31. PBa carried out the experiments with help from FKJ. PBa and TB carried out the data analysis, and PBa, TB, and DO conceptualised the study.

Summary

The zebrafish possesses a highly complex and utilised visual system. Its input is comprised of four distinct cone types as well as one rod type. However, in larvae, rods are thought to be immature. Accordingly, in their larval form, all visual input to the retina and brain circuits comes from the four cones. These feed into morphologically and functionally distinct and highly diverse circuits to ultimately drive a wide array of visual behaviours. While spatio-temporal processing in larval zebrafish has been studied at considerable depth, comparatively much less is known about their spectral processing. The goal of this thesis was to systematically map the physiological responses of most visual neurons in larval zebrafish – from cones via bipolar and ganglion cells to brain neurons - to stimuli that vary in wavelength. Specifically, we used 2-photon Ca^{2+} imaging of light-driven activity both in the retina and the brain (Chapters 3,4,5). We used an original 2-photon microscope modification to allow for fast multi-plane imaging (Janiak et al, 2019). Stimulation was carried out using a custom-built high-speed monochromator (Belusic et al, 2016) with a high spectral resolution (Chapter 2). Transgenic lines expressing Ca^{2+} -sensors in specific cell populations were generated to selectively observe different neuron types. We imaged cones and bipolar cells in the eye, and ganglion cell terminals as well as the somata of central neurons in the brain. Together, this served to establish a large-scale overview of the spectral computations that are performed at each stage, and how they may aid zebrafish visual functions.

In the six chapters of this thesis I:

1. Introduce colour vision in the zebrafish.
2. Describe the construction of the visual stimulator.
3. Describe and discuss the photoreceptor data. Cones transform chromatic signals in a Principal Component Analysis-like manner. This can be part-explained as an adaptation to the spectral characteristics of the visual world (Zimmermann et al, 2018). Previously, such a transformation was thought to occur first in downstream circuits.
4. Describe and discuss the bipolar cell data. Synaptic terminals form several functional clusters that are highly wavelength-dependent, enabling possibilities for complex spectral coding. Spectral opponency is observed in several clusters. Moreover, eye-wide regional specializations are observed, in agreement with prior reports (Zimmermann et al, 2018). I conclude by discussing the functional layering of the inner plexiform layer of the retina in the context of predicted functional wiring from cones.

5. Briefly describe the ganglion cell axonal and brain somatic data. Unlike bipolar cells, their responses are surprisingly uniform. I hypothesize that the response profile is uniquely sensitive to near objects based on data from hyperspectral imaging.
6. Summarise the findings in light of the wider literature. I speculate about the overarching goal of colour vision, relate the findings back to Wilkins & Osorio (2019) and certain logic-related considerations.

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Abbreviations

Ab: Antibody

AC: Amacrine Cell

α : Cone Specific Absorbance

B: Blue, short wavelength sensitive opsin or cone, SWS2

BC: Bipolar Cell

C: Cluster

cGMP: cyclic GMP

dpf: Days post fertilisation

G: Green, medium wavelength sensitive opsin or cone

GC: retinal Ganglion Cell

GCaMP: GFP-Calmodulin-Myosin Protein

GFP: Green Fluorescent Protein

γ : Quantum efficiency

INL: Inner Nuclear Layer

IPL: Inner Plexiform Layer

kHz: kilohertz

LED: Light Emitting Diode

L-Opsin: Long wavelength-sensitive opsin

MOM: Movable Objective Microscope

ms: millisecond

μs: microsecond

μm: micrometer

NMF: Non-negative Matrix Factorisation

nW: nanoWatt

OPL: Outer Plexiform Layer

OS: Outer Segment

OSA: Outer Segment Area

PC: Principal Component

PCA: Principal Component Analysis

PMT: Photomultiplier Tube

PR: Photoreceptor

PSF: Point-Spread Function

Pts: points

PTU: 1-Phenyl-2-Thiourea

PWM: Pulse Width Modulation

QI: Quality Index

OOi: On-Off index

R: Red, long-wavelength sensitive opsin or cone

RF: Receptive Field

ROI: Region of Interest

RPE: Retinal Pigmented Epithelium

SPCA: Sparse Principal Component Analysis

s: second

SD: Standard Deviation

SNR: Signal-to-Noise Ratio

SWS1: Short wavelength-sensitive 1 opsin

SWS2: Short wavelength-sensitive 2 opsin

Tg: Transgenic

uv: Ultraviolet

UV: Ultraviolet

UV-opsin: UV-sensitive OpsinSWS

1. General Introduction

*And then black night. That blackness was sublime.
I felt distributed through space and time:
One foot upon a mountaintop, one hand
Under the pebbles of a panting strand,
One ear in Italy, one eye in Spain,
In caves, my blood, and in the stars, my brain.*

Vladimir Nabokov, Pale Fire

Introduction

Our sense of smell does not allow us to smell a thing in the neighbouring street, audition does not permit to hear past a few kilometres. But we can see the light of stars long dead. There is a level of structure in the world that is only knowable through vision and through no other sensory modality. This correspondence between facts of the world and propositions put into one's mind through vision is the reason the study of vision is desirable. One may object that zebrafish do not habitually stargaze. Vision still is a rather remarkable sense.

Zebrafish as a vision model

The zebrafish is a shallow-freshwater inhabiting fish. Adults grow to about 2-3 cm in length, but their more intensely studied larvae at 6-9 days post fertilisation (*dpf*) are ~2-3 mm in length. Zebrafish have very large eyes compared to their bodies, with larvae holding approximately half of all their neurons within their two eyes. This fact points at the import of vision to the animal.

Zebrafish possess four distinct cone types, defined by their principally expressed opsin: long wavelength sensitive (LWS, "Red"), mid- (MWS, "Green"), short-2 (SWS2, "Blue"), short- (SWS, "Ultraviolet, UV") - (I shall use these nomenclatures interchangeably). Variations of the Green opsin expressed are frequent; of those there are at least three kinds (Takechi & Kawamura, 2005), but we will disregard that for the current investigation. Functionally, this is one more type than humans possess (e.g. Robinson et al, 1993, Boynton, 1988). Additionally, in adult zebrafish LWS and MWS cones grow together over time to form double-cones. Hence, we call humans "cone trichromats" and zebrafish "cone tetrachromats". Four cone types make the zebrafish inherently interesting to a student of vision. Their shallow water habitat is permeable to ca. 75% of the light that reaches the ground (e.g. Levine & MacNichol, 1982), and zebrafish have been shown to react to highly complex visual features, including other zebrafish (Neri, 2012). Accordingly, we may expect to find intricate chromatic processing in the retina of this animal.

Sensation of light

Experience of visual stimuli differ in 3 major domains: 1. Time 2. Space 3. Chromaticity. I will follow with a discussion of chromaticity as it is most directly linked to spectral processing and colour vision, gently touching the two other domains where appropriate.

Light possesses the property of wavelength. In humans, different wavelength composition of light elicits different subjective colour sensations.

Upon incidence with objects in the world light – for example from the sun - is absorbed, scattered and reflected in a wavelength-dependent fashion. Light incident on animal visual organs is focussed, filtered and otherwise changed by the optics of the eye (e.g. discussed in “Animal Eyes”, Land & Nilsson (2013)). Some of the light is absorbed by chromophores attached to visual opsins inside photoreceptors. Visual opsins are proteins that absorb certain wavelengths better than others due to their structure and change their conformation upon absorbing a certain amount of energy (discussed later). The systematic differences in absorption probability as a function of wavelength is known as the absorption spectrum of an opsin (Fig 1.1).

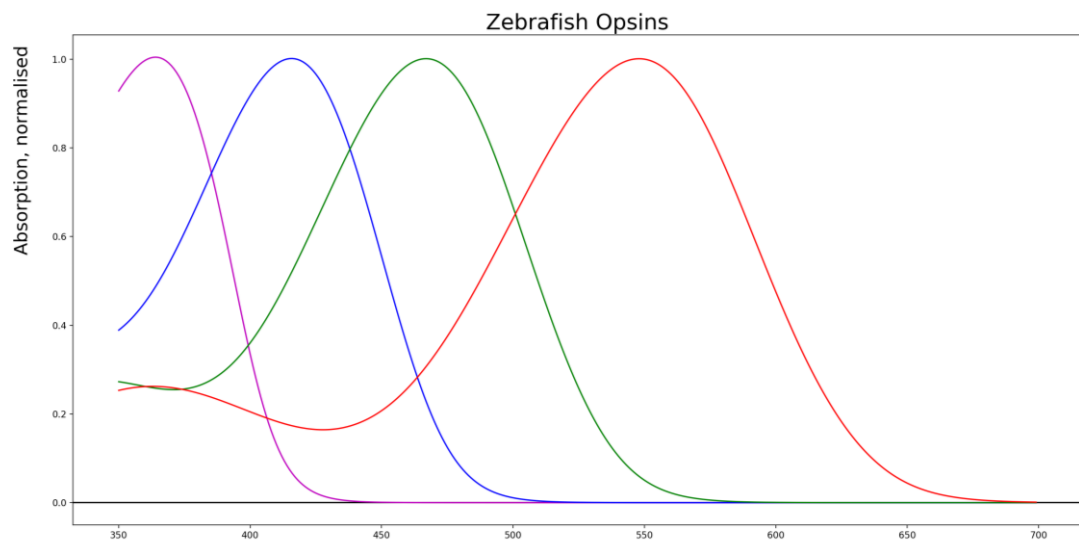


Fig 1.1 Zebrafish opsins' normalised absorption spectra

Govardovskii templates are fitted to the peaks as follows: Red - 548, Green - 467, Blue - 416, UV – 365 (Govardovskii et al, 2000).

Opsins are situated within the photoreceptor (PR) cells of the eye. I will use the terms “cone” and “PR” interchangeably unless otherwise specifically stated. An opsin’s absorption spectrum is linked to the structure of the opsin and the properties of the amino acids surrounding the chromophore. This structure is to a good degree predicted from several associated genes expressed within the PR cell. The PR nomenclature typically combines the name of the chromophore (A1, A2) and the opsin gene (varies) expressed. Absorbance measurements of dissociated zebrafish cones are presented in the table below (Allison et al, 2004).

Photoreceptor type	Mean $\lambda_{\max}$ in nm $\pm$ SD ^a (N)	Mean HBW ^b in $\text{cm}^{-1} \pm$ SD (N)	Difference in HBW ^c (cm^{-1})	Mean $A_{\max} \pm$ SD (N)	Mean OSD in $\mu\text{m} \pm$ SD (N)	Specific absorbance ^d (μm^{-1})
<i>Rods</i>						
Control (A ₁ -based)	503 $\pm$ 5 (194)	4007 $\pm$ 743 (106)	378	0.019 $\pm$ 0.0006 (186)	2.4 $\pm$ 0.3 (53)	0.0079
TH-treated (A ₂ -based)	527 $\pm$ 8 (212)	4385 $\pm$ 680 (212)		0.015 $\pm$ 0.0007 (160)		0.0063
<i>Cones</i>						
<i>LWS-double cones</i>						
Control (A ₁ -based)	565 $\pm$ 10 (14)	4109 $\pm$ 258 (14)	55	0.019 $\pm$ 0.0004 (14)	2.6 $\pm$ 0.3 (16)	0.0073
TH-treated (A ₂ -based)	613 $\pm$ 11 (20)	4164 $\pm$ 492 (20)		0.014 $\pm$ 0.0006 (15)		0.0054
<i>MWS-double cones</i>						
Control (A ₁ -based)	482 $\pm$ 6 (9)	4291 $\pm$ 128 (8)	261	0.015 $\pm$ 0.0005 (9)	2.4 $\pm$ 0.2 (12)	0.0063
TH-treated (A ₂ -based)	504 $\pm$ 19 (3)	4552 $\pm$ 198 (2)		0.014 $\pm$ 0.0006 (3)		0.0058
<i>SWS-single cones</i>						
Control (A ₁ -based)	411 $\pm$ 5 (31)	4478 $\pm$ 665 (29)	967	0.019 $\pm$ 0.0006 (31)	2.8 $\pm$ 0.8 (8)	0.0070
TH-treated (A ₂ -based)	412 $\pm$ 4 (4)	5445 $\pm$ 616 (4)		0.014 $\pm$ 0.0006 (4)		0.0052
<i>UVS-single cones</i>						
Control (A ₁ -based)	361 $\pm$ 3 (6)	n/a ^e		0.021 $\pm$ 0.0005 (6)	3.9 $\pm$ 0.3 (6)	0.0054

HBW, Half-band widths; $A_{\max}$, maximum absorbance; OSD, Outer segment diameter; TH, thyroid hormone; LWS, MWS, SWS, & UVS are long-, medium-, short-, & ultraviolet-wavelength-sensitive cones, respectively.

Fig 1.2 Light absorption characteristics of adult zebrafish cones.

Adapted from Allison et al, 2004. Mean $\lambda_{\max}$ corresponds to the peak of the opsin absorption spectrum.

Cones are divided into the inner and the outer segment. The outer segment hosts the opsins. Opsins are situated within the reticular formation, firmly held in place.



Fig 1.3 Mouse cone outer segment (OS).

Adapted from Carter-Dawson & Lavail (1979). The photoreceptive proteins are held within the reticular structures of the cone outer segment. EM close-up of a mouse cone.

As remarked upon previously, opsins change their conformation upon absorbing energy. Photon wavelength is a one-to-one function of photon energy. Hence wavelength tuning is energy tuning. The conformation change decouples them from the surrounding structure. This triggers a series of biochemical events that ultimately results in 1. A decrease in intracellular calcium within the cone inner segment. 2. Decrease of the PR membrane potential. 3. Decrease of release of the neurotransmitter Glutamate into the tripartite Cone-Bipolar Cell-Horizontal Cell synapse. 1.2.3. are all aspects of a “response” of a PR (Pugh & Lamb, 2000). This response is what we shall concern ourselves with in the next section.

Photoreceptor response

To relate the delivery of a photon to a PR response, we must consider the following:

1. A photon incident upon the sensory organ has a certain probability of arriving at the opsin.
2. It will either be absorbed or not with a probability dependent on its wavelength.
3. Absorption of the photon will trigger a conformational change in the protein with a probability that can be estimated experimentally (Dartnall 1968, $p=0.67$).
4. The protein detaches itself from the reticular formation and inhibits the Ca^{2+} -dependent Glutamate-release cascade. This happens via G-protein transducing decreasing the cGMP levels within the cell. This leads to hyperpolarisation of the PR membrane via cGMP-gated ion channel closure (for a reference, see Purves et al, 2004). Discussion of the protein machinery is not the subject of this thesis.

This response is further transmitted down the visual system.

A brief definition of core principles and terms

Let me establish the terms which I shall henceforth employ to account for changes in neurons' synaptic output associated with presentation of light stimuli to the sensory organ. This is done to distinguish between correlation and causation further in the text. I also distinguish between phenomena requiring explanation and not.

I shall say that a stimulus S **causes** X if X changes its value when S changes its value AND there is a known causal path (e.g. the PR response is caused by absorption of a photon) OR all possible causal structures have a directed path between S and X (example to follow).

Any change in the output of a cell A which is caused at least in part by some other factor B I shall call “**observation of B by A**”.

The dependence of response of a neuron on the wavelength of light incident on the sensory organ is referred to as a neuron's “chromatic response profile”, “**chromatic tuning curve**” or “spectral receptive field”. This nomenclature is agnostic of the causal path through which incident light causes a response within the neuron.

A Horizontal Cell or a Bipolar Cell directly observes only the present state of activation of a PR which they synapse with and each other's. Wavelength of the photon that caused the response in a PR is not directly observed by non-PR and PR cells alike.

Already at this point we arrive at an important distinction in our discussion: as stated previously, PR's probability of photon absorption is a function of photon wavelength. The “identity” of a photon as being of a particular wavelength or “perceptual chromaticity” is not **represented** within the PR. In other words, 2 photons **absorbed** by the PR with $P_{\text{abs}} = 0.5$ will have on average the same effect upon the response of the PR as 2 photons with $P_{\text{abs}} = 1$ (given that they are absorbed, - not to be confused with the probability of absorption of both 0.5 photons, which would be 0.25). This is known as the “Principle of Univariance”. But: given two PR's whose chromatic absorption spectra are different AND whose incident photons are plentiful; one can take the ratio of the activations of the two PR's. This ratio is revealing of the wavelength of incident light.

Given that 1. light of wavelength X caused Response Y in non-PR cell Z AND 2. the chromatic properties of incident light can be circumscribed by activation ratios of neurons causally upstream of Z; one can say that Y *in some sense* **represents** the chromatic property X.

In principle, this pyramid of neurons taking ratios between preceding neurons can be continued endlessly; arriving at infinitesimal precision of wavelength identity of incident light, given enough space for the neurons and uniform illumination.

Does it happen? If not, why? Possible answers to these questions can be glimpsed from section **“What is colour vision good for?”** and the corpus of this thesis. Foreshadowing, the distinction between observer and cell metamers will prove instrumental.

Further to convention: response profiles of many cells were observed; many of them cluster together. This clustering **defines** a functional type for the purposes of this discussion. Existence of different types I shall call **“interesting”**. Interesting things have to be remarked upon.

I shall use the words **“suggest”**, **“appear”** as “readily lets itself be interpreted as, recognized as”. This I will do in the same sense as: that looking at the sky **suggests** that the Sun revolves around the Earth. It is not a statement of fact, it does not indicate causation or logical following. (That it also looks like the Earth revolves around the Sun is a matter outside of the current investigation).

I shall use the word **“show”** as “recognizable and true, not needing further proof”. This usually is in the context of combination of empirical data and a strong assumption or some otherwise true statement.

I shall use the word **“verisimilar”** as “explaining a lot of empirical data; successful” when referring to theories.

Structure of the visual pathway

Zebrafish larva

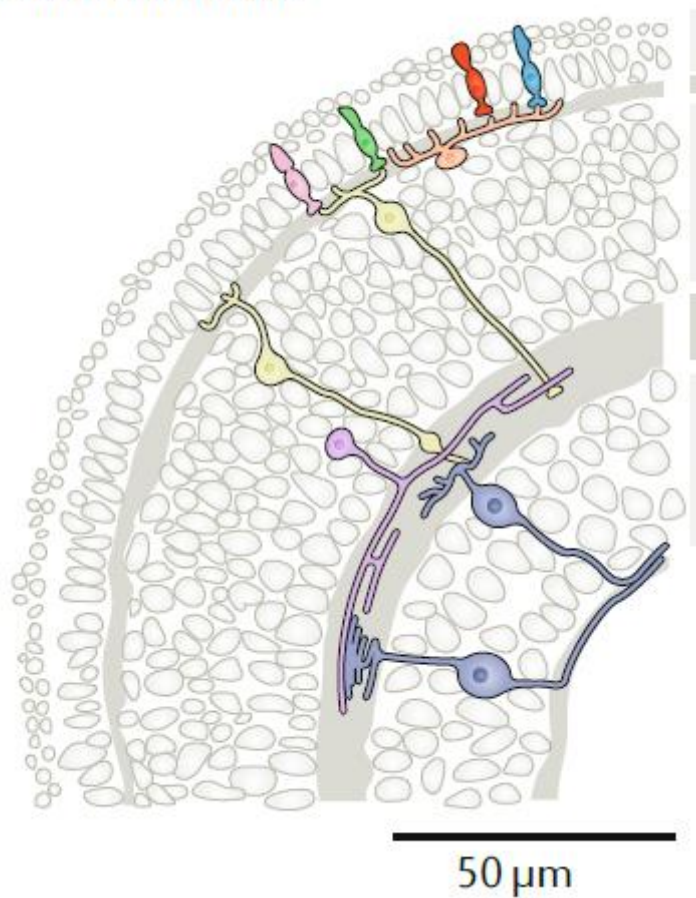


Fig 1.4 Neuronal architecture of larval zebrafish retina, schematic.

Adapted from Baden et al (2020). Light Magenta, Green, Red, Light Blue – UV, Green, Red and Blue cones, respectively. Light Red – Horizontal Cells, Yellow – Bipolar Cells, Magenta – Amacrine Cells, Dark Blue – Ganglion Cells.

The visual pathway is all the neurons of the retina and some of the brain. Many neurons represent visual information. Some of these neurons produce motor and premotor commands. These we explicitly exclude from discussion for pragmatic reasons.

PRs are untypical neurons, because they release neurotransmitters gradually and continuously. As mentioned previously increment in light stimulus produces decrease of neurotransmitter release. This allows the PRs to precisely signal a range of light levels. PR axons possess specialised neurotransmitter release sites, called ribbons. The signalling is both mechanically and functionally different to that in spiking neurons (e.g. Yoshimatsu et al, 2019). Despite this, they are principal neurons. Such principle

neurons are rare within the brain (another example is the Bipolar Cells of the retina, Meier et al, 2018).

As mentioned previously, PRs connect to Horizontal Cells and Bipolar Cells. BCs are principal neurons with gradual neurotransmitter release. HCs are interneurons feeding back to cones. HCs interconnect via gap-junctions. Thus average light levels across a relatively large patch of the retina can be represented in HCs. They proportionately inhibit the PR response (e.g. Masland, 2012). I do not know whether it has been experimentally shown, but it stands to reason that this subtraction of neighbouring cone activity from the activation of each individual cone produces an RF similar to the classical centre-surround response (as in Marr, 1982).

PRs, HCs and BCs together form the unique structure known as the tripartite synapse. Previous studies indicate that there are at least two temporally distinct types of inhibitory feedback from HCs (Thoreson & Mangel, 2012). Hence, BCs are expected to have a more complex temporal code than the PRs *ab initio*, indeed showing both sustained and transient responses to light. BCs are of two primary functional types: ON and OFF. ON BCs invert the PR activation polarity and depolarise in response to light. OFF BCs, on the contrary, hyperpolarise. This difference is due to expression of different Glutamate Receptors (GluRs) in these cells. ON BCs express metabotropic GluRs, that decrease inward Na^+ current following activation. OFF BCs, on the other hand, express ionotropic GluRs (AMPA and kainate). These depolarise the BC when the preceding PR depolarises. (see Euler et al, 2014; e.g., Ghosh et al, 2004, DeVries & Schwartz et al, 1999). Due to specificity of their PR connections, BCs form the classical colour-opponent centre-surround RFs (e.g., Wong & Dowling, 2005), These are usually thought to be the first colour-opponent cells.

BC synapses to PRs are in the Outer Plexiform Layer. BCs synapse and project to the Ganglion Cells and the Amacrine Cells. These synapses are situated in the Inner Plexiform Layer. Both HC and BC somata are in the Outer Nuclear Layer. BCs stratify into two distinct layers – ON and OFF by convention. This lamination is preserved and somewhat modified in the zebrafish larva (Zimmermann et al, 2018). It should be noted that there are various ways of classifying neurons into types; and we shall remark upon that in the course of the investigation.

Amacrine Cells are interneurons that modulate the BC and Ganglion Cell responses. Ganglion Cells (GCs) are the first principal neurons of the visual pathway that primarily spike. They are the only output from the retina to the rest of the brain via the optic nerve. Their axons terminate primarily in the tectum, though afferents are sent to other

brain regions as well (Robles et al, 2014). GCs process different types of features depending on their type. Progressively, the responses of retinal neurons become more and more feature-specific (e.g., Masland, 2012). The tectum is comprised of the large neuropil body and the carcass of neurons around it. This is a highly visual area. It is shown by various studies showing representation of different visual modalities (e.g., Niell & Smith, 2005).

It is the nature of this representation that I will be further discussing.

Chromaticity and contrast

Since every retinal point perceives itself, so to speak, as above or below the average of its neighbors, there results a characteristic type of perception. Whatever is near the mean of the surroundings becomes effaced, whatever is above or below is disproportionately brought into prominence. One could say that the retina schematizes and caricatures. The teleological significance of this process is clear in itself. It is an analog of abstraction and of the formation of concepts.

Mach 1868, in Ratliff 1965: 306

Human colour vision enjoys a remarkably simple verisimilar model: Hering's theory of colour opponency (Hering, 1920). Most humans are trichromats. In human literature trichromacy can refer to cone trichromacy and/or colour trichromacy. Humans are both cone and functional trichromats: humans possess three morphologically distinct types of cones and three primary colours given in sensation and spanning their perceptual space.

Human sensation includes non-spectral colours. These lie on a line between spectral violet and spectral red. I have perceived UV spectra as bluish milky white during my investigations. However, since we cannot ask zebrafish about their sensations, perceptual spaces are of particularly limited use for neurophysiological investigations.

One can define colour responses without appealing to the names of colours in different natural languages. Since first psychophysical investigations we know of the psychometric curve, where by definition a response is elicited by a Just Noticeable Difference in the stimulus (described for chromaticity by Judd in 1932). Such stimulus alterations are defined by contrast, that is, the ratio of the stimulus increment or decrement to the stimulus value. With neurophysiological measurements we could use the same language. Stimuli corresponding to particular "colours" can then be defined in terms of their wavelength composition.

Due to the "Univariance Principle" the organism must resort to contrasting different

cone responses, as discussed previously. Hence, the ratios of PR responses are expected to be a cause of colour vision. Indeed, human opsin-based models have been successfully used in numerous investigations of colour vision. Opsin-based models correspond to the Hering's opponent theory.

Chromatic contrast implies subtraction of one cone signal from the other, as discussed before. This phenomenon is known as opponency. Many neurons in the primate and fish visual pathways have exhibited colour-opponent responses (e.g. Dacey, 1999). Indeed, if contrast and chromaticity are represented in the visual system, **there will be corresponding neural structures**. This point simply follows from the doctrine of physicalism.

Given: 1. Cone ratios corresponding to colour sensations. 2. Fewer types of cones than minimal divisions of wavelengths; we expect many different spectra to produce the same cone ratio responses; and, as a result, to not be distinguished by the organism. This is known as metamerism.

Metamers and the “Silent Substitution” method

Metamers are different chromatic spectra such that *ceteris paribus* they 1. Elicit the exact same colour sensation in the observer, OR 2. Elicit the same pattern of neuronal responses. In other words, different physical stimuli elicit the same response in the receiver, however defined.

Estevez & Spekreijse (1982) popularised a system of linear equations (Silent Substitution) that relate three stimuli of different wavelength composition, and contrast-response of a system comprised of three differently-tuned receptors. In other words, the experimenter can achieve a certain chromatic contrast by manipulating the strength of three light-sources of different wavelength compositions. As an example, equal cone activations can be elicited with different tristimulus inputs. This system is naturally extended to more receptors and light sources. The stimulus and response can be related with the relations outlined in the **PR response** section. We derive such a system for the zebrafish in Franke et al (2019).

To elaborate on this: the method relies upon the correctness of Grassman's laws. Two tristimuli (stimuli constituted by three colour primaries, ~ corresponding to the three receptors) are considered metameric if the presented stimuli are different but the response of the receptor system is the same. If A is a metamer of B then B is a metamer of A. Further to that, colour matches, which metamers are a special case of,

are both additive and commutative. All of these laws mean that one can solve for metamers using a simple system of linear equations. Negative values are a convention when some tristimulus is defined with respect to a non-zero reference tristimulus.

By extension, through the property of additivity of the tristimuli, one can parameterize the equations as functions of wavelength, as opposed to predefined primaries. This yields: $R(\lambda)$, $G(\lambda)$ and $B(\lambda)$ for responses of the three chromatic (or physiological) cones as functions of wavelength. As long as the response properties are sufficiently linear, the stimulus vector is translated into the response vector via matrix multiplication, where the elements of the matrix denote the effect of stimulus basis x_i on some receptor y_j in units of Y per unit of X (e.g., released neurotransmitter per lumen), yielding the matrix M . Required response multiplied by M^{-1} yields the required stimulus. In its algorithmic essence, the Silent Substitution procedure is solution of a system of linear equations via matrix multiplication.

Consider the vector of stimuli s , the response vector r and the matrix M describing the mapping s to r .

$$s \times M = r$$

The possibility of metamers with respect to a given r_a is equivalent to saying that s_a such that $s_a \times M = r_a$ is a set of vectors with more than one member other than $\emptyset$. With a greater number of light-sources than receptors the solution for a given contrast is not unique.

However, recently Kamar et al. (2019) measured current and voltage responses of cones to metameric stimuli defined in the above sense. This revealed that stimuli that were metameric for voltage responses were not metameric for current responses. Accordingly, the Silent Substitution parameterisation of the stimulus might not be what we are looking for in a neurophysiological investigation.

Hence there is inherent indeterminacy in the visual stimulus and physiological response relationship. So, what sets of stimuli are preferentially mapped onto the responses of the colour vision system?

What is colour vision good for?

Here I shall deliberate upon the first principles which we ought to concern ourselves with to place the present thesis firmly upon the shoulders of giants.

PCA and colour

No contemporary student of colour vision can overlook the Ruderman & Cronin (1998) “Statistics of cone responses to natural images: implications for visual coding”. Images were drawn from “natural” environments for humans. The Principal Component Analysis (PCA) algorithm was applied to them. This operation is of such high import for the study of colour vision that it will pay off to further deliberate on it.

There is no negative light in the world. Any contrast must be made relative to defined level somewhere in the non-negative region of light reception. The ON and OFF pathways are well-suited to do just that. Moreover, reflectance spectra in the world are autocorrelated in the wavelength domain (if they were not, chromatic vision would not have been possible). The concept of colour vision partially accounts for contrasts we see in the world. If there were no spectral differences in the observed world, it would have been made of one material. Colours would not exist in any familiar sense of the word.

PCA finds the longest projection vector for variance vectors in the data. The data are then rotated in accord with the projection and the new longest projection vector is found, exhaustively. When applied to the spectral domain of natural images, the first such vector will be strictly non-negative *in absentia* noise in the measurement – in practice usually strongly resembling the mean spectrum of the scene. Further orthogonal components will cross the original abscissa.

Up until very recently PCA has primarily been carried out with human or primate opsin responses. The human opsin responses to natural images are highly correlated. The first PC will necessarily be some sum of non-negatively weighted opsin responses (positively weighted if there is any light in the image of the wavelength that the opsin is sensitive to). Further PCs exhibit contrasts between L+ and M- wavelengths; and L+M-S+ wavelengths. The wavelength regions correspond to spectral colours that contrast perceptually (e.g., Buchsbaum & Gottschalk, 1983).

Due to information-theoretic concerns explicit representation of chromatic signals in these terms is efficient (e.g., consider the classical example of “pyramid code” in Burt & Adelson 1983). This seems like a natural principle to govern wavelength hotbeds of opponency. Indeed, data from primates (e.g., Solomon & Lennie, 2007) and zebrafish alike (Zimmermann et al, 2018) seem to follow this prediction.

In Ruderman et al (1998), the achromatic component (first PC) accounted for over 90% of variance in their dataset. The first two “colour” components (PCs 2 and 3) accounted

for 4% and 2%, respectively. However, if **information about pixel intensity** variance in the image is the criterion of a system's success; it may be considered strange to find that a disproportionate number of retinal and brain cells have chromatically-contrasting responses (e.g., Kolb et al, 1985). In addition, Li et al (2002) observed that human cone PR sensitivity peaks are not optimal by this measure. What, then, causes the “colour” structures of the brain to be as they are?

Is the world colourful?

Es ist eine erstaunliche Unwahrscheinlichkeit, dass wir auf der Erde leben und Sterne sehen koennen, dass die Bedingungen des Lebens nicht die des Sehens ausschliessen oder umgekehrt.

Hans Blumenberg, Die Genesis der kopernikanischen Welt.

It is most unexpected that we live on Earth and have the ability to perceive the stars; that the circumstances of life do not preclude this unique vision.

Hans Blumenberg, The Genesis of the Copernican World (own translation)

There is a tradition of thought that explains sensation via facts of the world and their relations. A broad discussion would have to include at least Aristoteles, Mach, Russel and Marr. Here I simply wish to highlight a recent instrumental article within this tradition.

Wilkins & Osorio's (2019) account is highly technical and concerns itself with “vividness” of visual space construction. I will produce here a summary to convey their particular discoveries.

Consider two spectra: one skewed to shorter wavelengths, the other to longer wavelengths. In a trichromatic system they will produce a response indicating chromatic contrast. Their mixture will produce a response with less chromatic contrast. In other words, the hue of the resulting response is closer to the origin of the space.

The spectra are facts of the world. They result to a great extent from the material which the light is incident on. The sampling of these distinct spectra across space depends on the effective spatial visual acuity of the animal. A lower spatial acuity results in a higher degree of mixing of spectra at the sampling level, so long as the observed object(s) are smaller than the receiver's effective receptive field. Larval zebrafish visual acuity is limited to 2 degrees of visual angle due to cone spacing. Psychophysically, it was measured to be around 3 degrees (Haug et al, 2010).

Consider metamerism. Metamerism is typically measured to be very low in natural images (e.g. Foster et al, 2007). However, this notion is inherently dependent on the observer model, i.e., metamers are defined with respect to a given set of receptors. The greater the number of receptors – the fewer metamers. A greater degree of spectral mixing results in higher metamerism levels and the mentioned shift to the origin. In other words, grey is defined as the hue with the most metamers. Conversely, “pure” spectra will produce contrast responses for which fewer metamers exist. In other words, purity of the material from which light is reflected into the eye converts to higher determinacy (or Shannon information) content of the response.

Naturally, the degree of metamerism also depends on the number of PR types. This can be seen as a corollary to the principle established in the **Metamers** section. Could therefore zebrafish tetrachromacy compensate for this animal’s lower visual acuity as compared to humans? This thesis will explore this possibility, which may go partway to explaining why so many neurons in diverse species’ eyes and brains are colour-opponent (e.g. Solomon & Lennie, 2007, Chatterjee & Callaway, 2003). In this view, **information about the nature of objects** is conveyed through chromatic contrast. An intuitive example of this kind of message is the “popping out” of fruits on leafy backgrounds that is often used to explain evolution of primate trichromacy (Regan, 2001). Larval zebrafish exhibit comparable foraging behaviour. Prey-capture responses are elicited by moving dots of light or dark contrast (Flamarique & Novales, 2013). UV chromatic signal is especially suited to elicit this response (Yoshimatsu et al, 2020, Famarique, 2013).

Interestingly, the authors indicate that three “vividness” axes are sufficient to efficiently represent information about materials **for any species**. By this account, cone tetrachromacy does not imply functional tetrachromacy on the entirety of the seen spectrum.

Noise and gain in PRs

Atick (1992) is another cornerstone in colour vision science. The author derives that summation of opsin signals results in a potentially higher-speed and higher-acuity visual channel than their subtraction. This is due to signal-to-noise ratios in the resulting channels.

From here, Vorobyev & Osorio (1998) proposed that colour discrimination thresholds in animals are to a large extent determined by **photoreceptor noise**. Their modelling shows little to no effect of post-PR neuronal noise on chromatic discrimination

thresholds. Their model is both simple and verisimilar.

Li (2002) then weaved in how cone PR potential sensitivity peaks are limited by thermal noise. Sensitivity to infrared is increased with the peak wavelength of the PR.

PR noise is diminished with summation and subtraction of PR signals. Multiplicative gain is needed to further propagate small signals. This gain will multiply noise and signal alike. Signal-to-noise ratio at the level of the PR is thus limiting possible contrasts.

Proposed BC pyramid (see section **A brief definition of core principles and terms**) and other schemes of producing more axes of chromatic contrast suffer from the PR noise limitation.

To effectively cancel out PR noise PR responses should be approximately equal. I assume that this is the strategy employed by the retina. Non-flat “natural” spectrum means that PR output gain differs as a function of PR peak wavelength. Endeman et al (2013) conducted intracellular voltage measurements in isolated zebrafish cones. The measured gains do not complement the natural spectrum. Interestingly the authors found that the maximal voltage response of the SWS2 cones was approximately half that of other cones. In accord with other investigations (Vasserman et al, 2013) I suggest that the zebrafish PRs adapt to natural environments. It was not measured by the mentioned authors.

Noise in the world

Maximov (2000) remarks that the underwater visual world is different from the overground one. Maximov argues that ripples on the water result in increased achromatic visual flicker in shallow water-inhabiting animals. Mechanisms of chromatic vision help edge detection under such circumstances. The achromatic flicker is a property of the illuminant. Objects have chromatic properties that are determined by the reflective surface. Objects can be distinguished on the basis of such properties. Multiple cone types produce contrast (in the above defined sense) due to these properties. Ceteris paribus this contrast does not depend on the mean brightness level. Hence chromatic contrast enhances object segmentation.

This is a particular example of the general principle outlined in section **Is the world colourful?** The specific relevance is to the zebrafish, which inhabits shallow water.

Hyperspectral data from Zimmermann et al (2018) will be further examined in the **Cones** section. Here, briefly: longer wavelengths are prevalent in the zebrafish visual

world across much of visual space (except when looking straight up), UV signals are about an order of magnitude weaker than the long-wavelength signals. This converts to lower signal-to-noise ratio in the shorter wavelength region.

Experimental Design

Taken together, these considerations left me with a simple experimental design.

We investigate the response magnitude of different neurons to light of different chromaticity. To show precise loci of opponency (see section **A brief definition of core principles and terms**) and due to metamerism concerns (**Metamerism and Silent Substitution**) I needed high spectral resolution of the stimulus.

Due to natural spectrum adaptation (**Noise and gain in PRs**) and indeterminacy of the greypoint (**Is the world colourful?**) I had no prior expectation about what the origin to which contrast is measured should be.

To observe transformations of the chromatic signal, more than one neuronal layer should be observed.

So, we stimulate Cones, Bipolar Cells, Ganglion Cells and brain neurons (Ch3-5) with flashes of light of highly restricted wavelengths. To this end, I first constructed a **Light Synthesiser** (Ch2).

We relate our findings to the spectral recordings of the zebrafish visual world throughout this thesis.

2. Light Synthesiser

Odours of incense came to match the golden notes; and overhead a great light dawned, its colours changing in cycles unknown to earth's spectrum, and following the song of the trumpets in weird symphonic harmonies.

Howard Phillips Lovecraft, The Dream-Quest of Unknown Kadath

Introduction

This chapter describes the construction and validation of a visual stimulator capable of delivering high spectral resolution visual stimulation (i.e, stimulation with a great number of distinct non-combinatorial spectra); the recording conditions; as well as the schema of the presented stimuli. This is important to establish the characteristics of the stimuli used in the chapters to follow.

We have established in **A brief definition of core principles and terms** that there is no *a priori* reason to think that a particular set of four illuminant spectra will allow one to effectively produce metamers for a given cellular layer or a given set of responses. That is, until one has established the organizational principles of the zebrafish visual system's wavelength response. We set out investigate such principles across several neuronal layers. We assume that rich chromatic response profiles from different neurons will imply a certain organisation. It follows that increased spectral resolution of the stimulation and of the response will provide useful data.

I adapted the design of Belusic et al (2016): "A fast multispectral light synthesiser based on LEDs and a diffraction grating". The described construction provides a cheap and easily adjustable visual stimulator with arbitrary spectra. In short, an array of LEDs of different peak wavelengths is arranged on an optical rail opposite a diffraction grating. The LED-emitted light diffracts off the grating at an angle that is a function of the light wavelength; in accord with the equation below:

$$\alpha(\lambda) = \sin^{-1}(G\lambda - \sin(\beta_{+1}))$$

where:

α – angle of the light incident to the diffraction grating,

λ - wavelength, nm,

β – first order diffraction exit angle,

G – diffraction grating groove density.

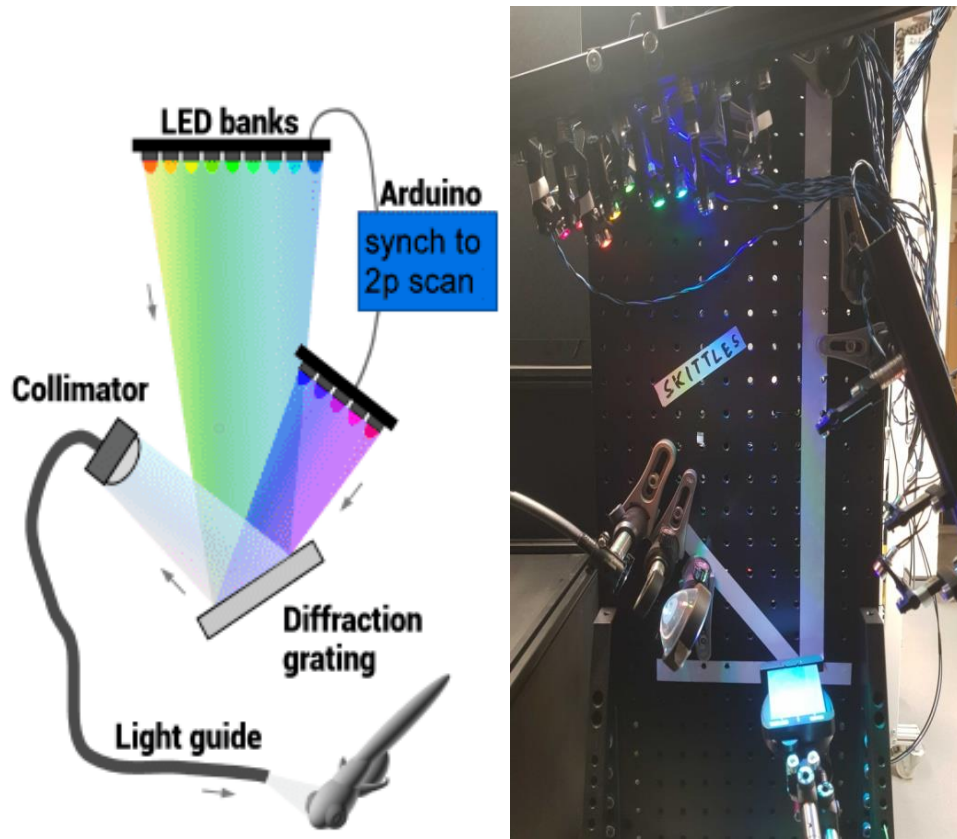


Fig 2.1 The light synthesiser: schematic and photograph.

Left: schematic

Right: photograph of a working setup. As seen: LEDs on two optical rails, diffraction grating and the light collection setup (focussing, collimating lenses and the lightguide).

Arranging the LEDs in a manner such that the emitted light impinges upon the grating at different angles, one can align the diffracted light beams together. The light is then focussed by a lens and collected with a spectrally broad liquid waveguide with a low NA (0.59) (77555 Newport). The other end of the waveguide is then directed at the eye of the experimental animal to provide for effectively full-field stimulation. Thus, with multiple LEDs visual stimulation of great spectral resolution is achieved. For further details see Belusic et al (2016).

One limitation in the principal design is that only a low fraction of each LED's emitted light is ultimately projected onto the sample. Accordingly, starting from the comparatively low achievable luminance design published by Belusic et al (2016), I undertook a number of optimisations with the goal of increasing effective stimulus brightness. The observed luminance is a function of the amount of light collected at the collection end of the fibre. However, in off-the-shelf non-collimated LEDs the emitted light greatly dissipates with distance. To therefore increase the amount of light collected I have pursued several

strategies:

1. Collimate the LED light, each LED thus requiring a collimator. The maximally achievable collimation strength is a function of the size of the collimator.
2. Bring the LEDs in closer apposition to the diffraction grating; thereby decreasing the amount of light 'missing' the grating. This strategy however restricts the amount of space available for the arrangement of the LEDs. Effectively this restricts the number of LEDs that can be placed.
3. Insert parabolic mirrors into the setup, to allow for much more flexible positioning of the LEDs. (suggestion of Dr F. Janiak)

Methods 1. and 2. were employed in the end. Each LED was put inside a collimator case. Following that I trialled various suitable spatial arrangements of LEDs before the diffraction grating. Diffraction orders 1 and 2 were used. In total, I fitted up to 17 LEDs, ranging from 359 to 656 nm in peak wavelength. These were positioned on two rails which allowed placing the generally weaker short-wavelength LEDs more closely to the diffraction grating.

The collimator cases were held and attached to the optical rail with custom 3D-printed holders (available at <https://github.com/BadenLab/HyperspectralStimulator>).

Spectra were measured using the Thorlabs CCS200 spectrometer at the output end of the lightguide. An example of achieved spectra can be seen in Fig 2.2. (over the course of the project, somewhat different LED arrangement were used before settling on the below version). This also highlights the versatility of the stimulator.

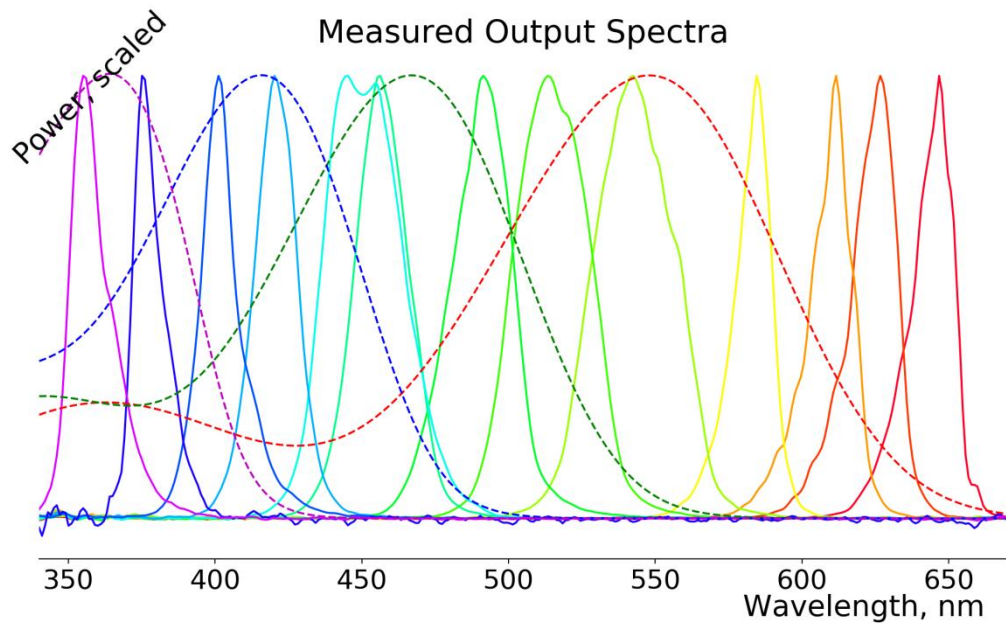


Fig 2.2 Stimulus spectra.

Measured at the output end, scaled to 1 at peak for presentation purposes. Dotted lines represent PRs, corresponding to colour: R, G, B, UV.

The output spectra are narrower than those typical of LEDs. This is due to narrowing of the incoming spectra through wavelength-dependent diffraction. Effectively, this meant more selective stimulation of the visual circuits.

One spectrum has an uncharacteristic “two-hump” shape. The angle of the diffracted spectrum is wavelength-dependent. The shape is due to partial occlusion of the LED-diffraction grating lightpath. This occlusion was necessary to arrange LEDs as close to the diffraction grating as possible. Indeed, Fig 2.1 shows that LEDs are arranged in several rails.

LED control

The experimenter controls upwards of ten LEDs. The LEDs are best individually regulated using Pulse-Width Modulation (PWM). Luminance of the LED is a non-linear function of input voltage, but with PWM one can achieve linearity in a large portion of the PWM-luminance function. PWM is the modulation of the duty cycle of current pulses supplied to the LED. Luminance output of the LED is approximately a linear function of the duty cycle in some range of PWM values. Such dependence is demonstrated in Fig 2.3.

Light Power as a function of PWM

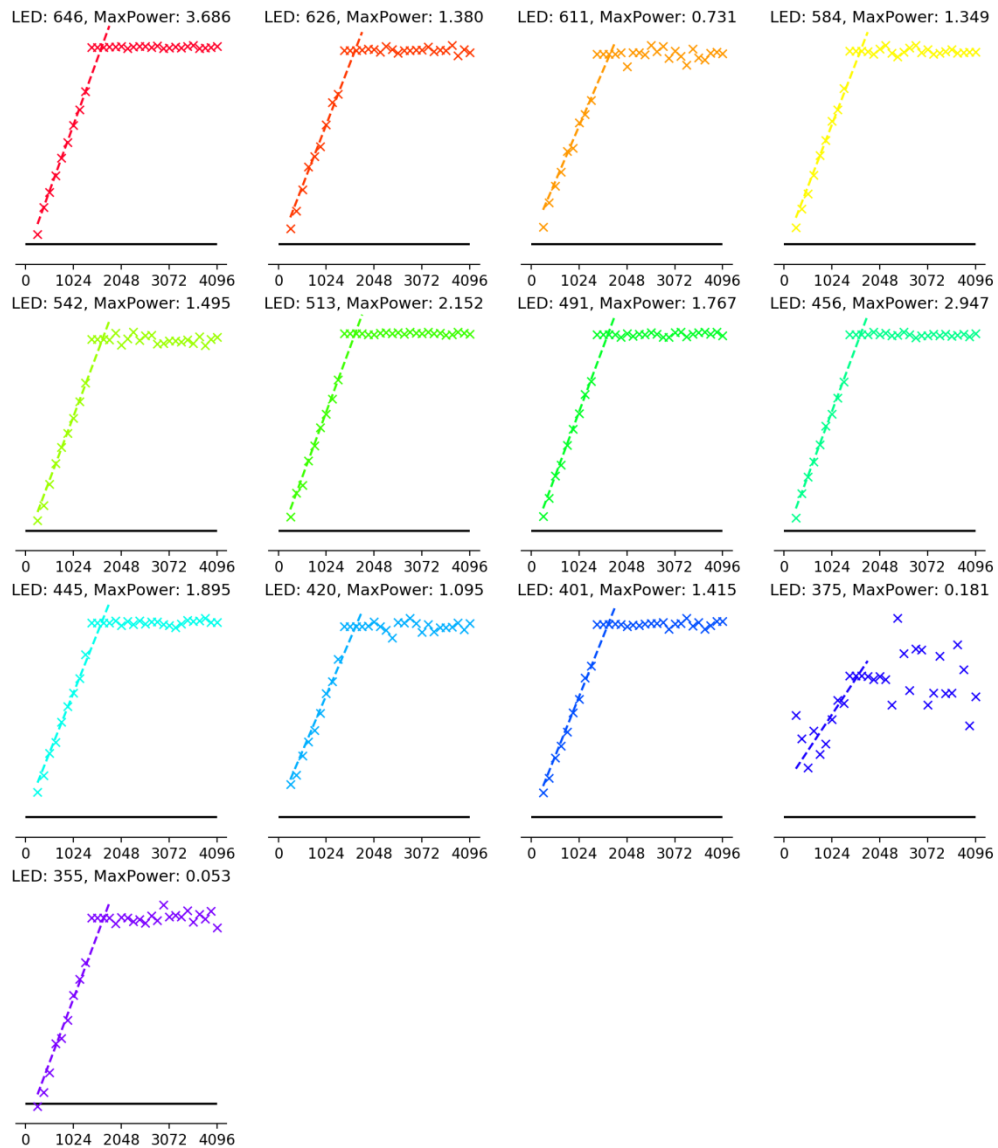


Fig 2.3 LED output vs PWM.

LED output as a function of Pulse-Width Modulation for a given set of LEDs. The linear portion of the PWM-power function is highlighted with a dotted line.

These dependencies were characterized with linear equations, one for each LED. Fig 2.3 shows plateaus at higher PWM values. Only the approximately linear portion of the current-light curve was used for stimulation. High PWM resolution of the driver still left me with a wide range of useful PWM values and thus effective brightness values. Several trialled LEDs changed their output spectrum with increased power. These were replaced and discarded in favour of more spectrally “robust” ones.

2-p laser coupling

I used an Arduino Due microprocessor (Arduino) with the Adafruit TLC5947 LED Driver (Driver) to drive the LEDs.

The LED Driver was used due to 1. its high digital pin resolution (12bit) giving an effective PWM resolution of 4096 possible values 2. 24 PWM pins allowing me to drive up to 24 LEDs.

We employ 2p scanning microscopy for physiological data collection. The data are recorded using photomultipliers (PMTs). PMTs detect very low light levels emitted by the excited fluorescent proteins. However, visual stimulation bright enough to effectively probe the cone-photoreceptor system of an animal typically produces enough incident light to generate stimulus artefacts in the scan, or to even overload the PMTs. Accordingly, we employed a time-separation strategy of scan epochs and stimulation epochs: As the scanning and data collection are taking place, the visual stimulus is off. When the visual stimulus is on, the data collection is not taking place (the principal strategy is reviewed in Euler et al. 2019). This switching back and forth has to occur at a sufficiently high rate that the visual system does not “see” the flicker – in case of the larval zebrafish this should therefore be at least several 10s of Hz, and ideally substantially above this. Here, we implemented the switching at 500 Hz, which is equivalent to switching the LEDs on once every 2p scan line.

For this, we read the digital signal produced by the scan software via its acquisition board (details) that varies between LOW and HIGH as a function of scan lines and retrace, respectively (dubbed the “blanking signal”). This signal was scaled, inverted and input to the “oe” pin of the stimulator’s Arduino Due. This pin overrides the power delivered to all output pins, and in this way conveniently ensured that the LEDs were rapidly switched off during the scan. Only during HIGH signal for the blank were the LEDs then permitted to be on, depending on their current PWM command. This is illustrated in Fig 2.4 (code available at BadenLab GitHub - <https://github.com/BadenLab/HyperspectralStimulator>).

There has to be a known and definitive coupling of the stimulus to the recording. The researcher has to know the state of the scan at a given timepoint. The timepoint is defined by the state of the Arduino that produces the light stimulus. I read the signal coupled to the stimulus into the imaging software (Trigger). For each timepoint in the recording the Trigger value was known. The relevant timing signals can be seen in Fig 2.4.

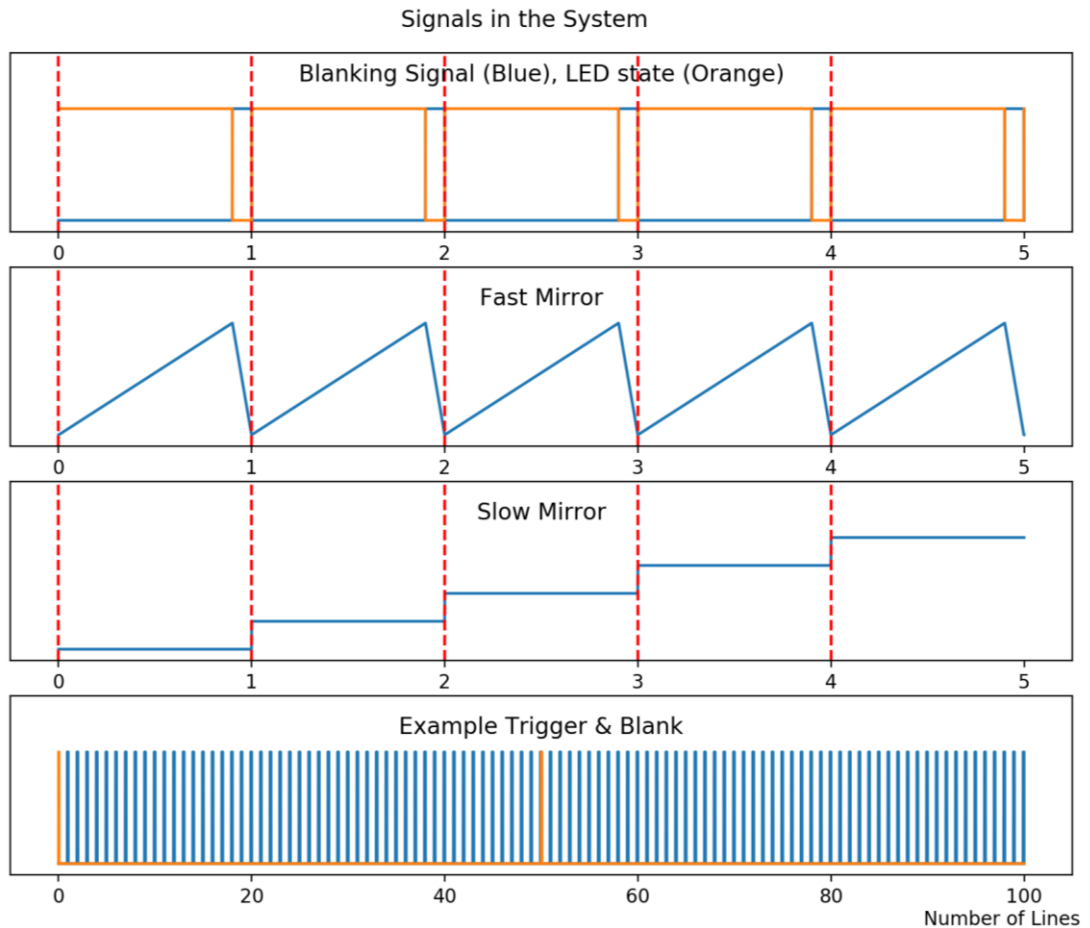


Fig 2.4 Electric signals in the microscopy setup.

Signals in the imaging setup that need to be aligned. Blanking signal is the inverse of the LED state.

The Fast mirror of the setup has to retrace every line. During the retrace no data collection takes place as mentioned before. During the retrace period the stimulus is shown. The slow mirror signal is irrelevant to the blanking procedure. It is related to the Trigger signal because the Slow Mirror movement dictates frame alignment within the software.

Two-photon Ca^{2+} imaging and light stimulation

Stimuli were presented to one eye. The output end of the lightguide is pointed at the zebrafish fixed in agarose. Between the fish and the fibre a spectrally-neutral Teflon diffuser was placed. The fish are sometimes sufficiently transparent for some light to reach the contralateral eye through the body of the fish.

We used a MOM-type two-photon microscope (designed by W. Denk, MPI, Martinsried; purchased through Sutter Instruments/Science Products). Design and procedures were described previously (e.g. Zimmerman et al, 2018). In brief, the system was equipped with a mode-locked Ti:Sapphire laser (Chameleon Vision-S, Coherent) tuned to 927 nm, a single fluorescence detection channel for GCaMP6f (F48x573, 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 6.3 for Windows (Wavemetrics), taking 3 128x64 pixel 1 ms per line image sequences (15.625 frames per s) for our BC terminal scans; 1 128x64 pixel 1 ms per line image sequences (15.625 frames per s) for our cone scans; 1 128x64 pixel 2 ms per line image sequence for the Anterior-Posterior and the Medial-Lateral axes of the tectum (7.8125 frames per s); and 3 160x350 pixel image 1 ms per line (6.25 frames per s) sequences for the whole-fish pan-neuronal recordings. The scan speed (1 or 2 ms per line) was manually adjusted to reveal different components of the response waveform while maintaining acceptable SNR.

We introduced key modifications to the previously used optical imaging system. The principal design is presented in detail in Janiak et al (2019). In brief, the optical arrangement was not infinity-corrected as is traditional in 2p scanning microscopy, but instead uses a non-telecentric design. As a result, the maximal field of view can be flexibly expanded, at a trade-off that also inflates the size of the excitation spot (point spread function, PSF) – effectively reducing maximal optical resolution but boosting signal integration from the excited area. In general, an optimum of this tradeoff can be achieved if the dimensions of the PSF and those of the neuronal target(s) (e.g. synapses / cell bodies etc.) are approximately matched. For experiments presented in this and the following chapter, the optical design was set such that the entire zebrafish brain could be imaged without moving the sample (field of view ~1.2), and the PSF was concurrently expanded to approximately match the dimensions of the “large” bipolar cell terminals (1-2 microns in diameter). This reduced oversampling in the xy-plane, at the cost of slightly increasing the risk of merging signals across the z-dimension

(because the PSF is inevitably z-elongated).

Moreover, an Electrically Tunable Lens (ETL) is introduced into the laser excitation light path prior to the scan mirrors to enable rapid (millisecond-range) z-focussing. The ETL's focal length at the centre is modulated via change of current flowing through the liquid in the ETL. We built a piece of electrical circuitry that transformed DC input from an Arduino board into acceptable AC ETL input. The Arduino control board was coupled with the Igor 6.3 Imaging software. This allowed us to manipulate current, diopters, and recording depth on every line of the scan. Thus, multiple quasi-simultaneous recording planes later highlighted in Figs. 4.1 and 5.6 are achieved with the goal of increasing the number of neuronal elements that could be surveyed at a time.

Spectrum Measurements

The measurements were carried out with the scan software running. The integration times were significantly shorter than those used for stimulation (ca.50-70ms). The measurement results rule out the possibility of signals being “blanked out” by the laser scanning software. These measurements were carried out using the Thorlabs CCS200 spectrometer at the point of output from the lightguide. Low visible noise levels as seen in Fig 2.2 are an indicator of significant output power achieved. The CCS200 spectrometer is only sensitivity corrected up to 365 nm. Some of the LEDs used had considerable power in the shorter wavelength range. To observe such LED spectra I derive a new correction curve using a broad spectrum deuterium lightsource with a known spectrum. Comparison of the default correction curve and the one derived by me can be seen in Fig 2.5

The new correction curve is derived by comparing the spectral measurements with the CCS 200 to spectral measurements taken with a physics-grade calibrated Maya spectrometer. The latter reports data as Power (in nW) and not Counts. In other words, it is wavelength-corrected. Hence, in the above (Fig 2.3) LED output is reported as Power.

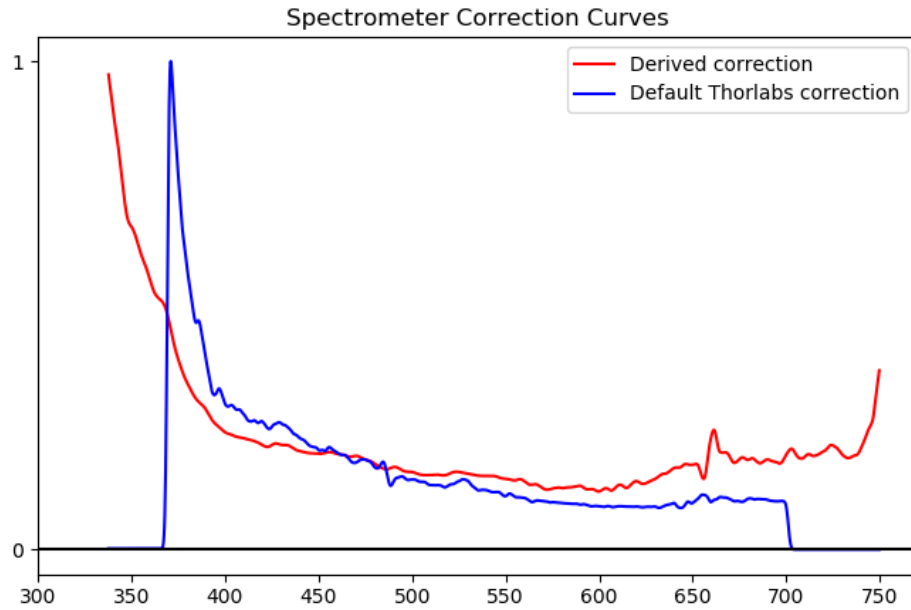


Fig. 2.5 Correction Curves

The default software correction (blue); the empirically-derived correction curve (red).

The increased gain in the shorter wavelength range is due to low detector sensitivity in this same range.

The Visual Stimulus

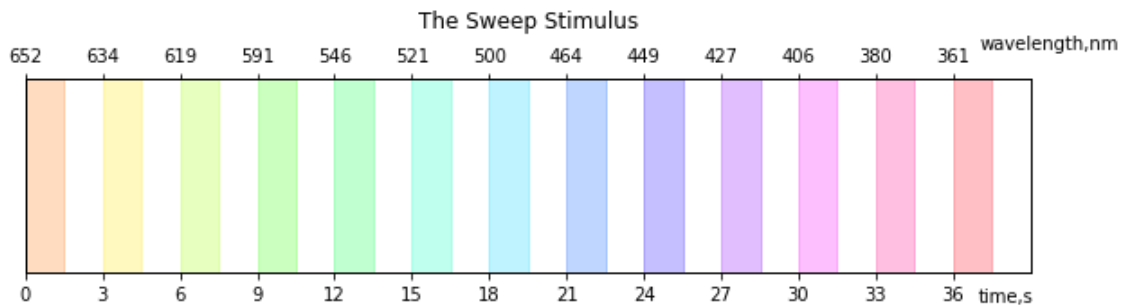


Fig 2.6 The visual stimulus schematic.

LEDs with indicated peaks were presented full-field consecutively, in the order shown. The timing corresponds to the stimulation time. The stimulus was repeated for at least three times.

The visual stimulus is conceptually the same for all layers of the visual system. The LEDs were switched on sequentially, from longest to shortest wavelengths. The LED presentations were interleaved with periods of no light being presented for the same amount of time. As follows: LED1 On – All LEDs Off – LED2 On – All LEDs Off – LED3 On - ...

The presentation time was adjusted for different neuronal layers by eye. As an example, the presentation time for Bipolar Cells is 1.5 s On and 1.5 s Off for a given LED. It is double that for isl2b and H2B data, namely, 3 s On and 3 s Off. This was done to consistently distinguish different response waveforms in different neural structures. Bipolar Cell Terminals' Ca^{2+} -response dynamics allowed us to distinguish between more sustained and more transient responses, for example. On the other hand, H2B responses are too slow and had to be further processed.

As noted previously, the light synthesiser was incrementally improved over time. Different experiments were carried out with different LED arrangements. The number and spectral peaks of the presented stimuli is noted in the figures of the corresponding sections. Where possible, I colour-code the spectral peak of the stimulus with colour bars.

Importantly, most of the LEDs are equalized in output power. As can be seen from Fig 2.3, maximal output power achieved with LEDs of wavelengths shorter than 400 nm, is significantly lower than that of other LEDs. The UV LEDs are equalized in power between themselves, and thus are an order of magnitude dimmer than other LEDs.

Initially, all LEDs are digitally equalised thus limiting the long-wavelength stimulus' power. Preliminary experiments showed that UV responses are overwhelmingly large compared to responses to other wavelengths in all neuronal layers except Cones and Horizontal Cells. Hence, for BCs and brain data, sub-400 nm stimuli are on average an order of magnitude less intensive than others.

Sidenote: *The stimulator was enhanced to produce chromatic centre-surround and moving stimuli. A system of lenses, and electronically-controlled mirrors was devised to that end. This work was presented in a poster at the 2017 European Retina Meeting. Part of that work contributed to Janiak et al. (2019). However, none of the data collected using this “spatial” version of the stimulator is presented in this thesis, which instead focuses on the full-field stimulus-response functions of retinal and brain neurons (Chapters 3, 4, 5).*

3. Cones: near-optimal rotation of the spectral signals

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Ancestral circuits for vertebrate colour vision emerge at the first retinal synapse

Takeshi Yoshimatsu¹, Philipp Bartel¹, Cornelius Schröder^{2,3}, Filip K Janiak¹, François St-Pierre⁴⁻⁶, Philipp Berens^{2,3,7} and Tom Baden^{1,2\$}

1, School of Life Sciences, University of Sussex, UK; 2, Institute of Ophthalmic Research, University of Tübingen, Germany; 3: Centre for Integrative Neuroscience, University of Tübingen, Germany; 4: Department of Neuroscience, Baylor College of Medicine, Houston, TX, USA; 5: Department of Electrical and Computer Engineering, Rice University, Houston, TX, USA; 6: Systems, Synthetic, and Physical Biology Program, Rice University, Houston, TX, USA; 7: Institute for Bioinformatics and Medical Informatics, University of Tübingen, Germany.

^{\$}Correspondence to t.baden@sussex.ac.uk

Abstract

For colour vision, retinal circuits separate information about intensity and wavelength. In vertebrates that use the full complement of four ‘ancestral’ cone-types, the nature and implementation of this computation remains poorly understood. Here, we establish the complete circuit architecture of outer retinal circuits underlying colour processing in larval zebrafish. We find that the synaptic outputs of red- and green-cones efficiently rotate the encoding of natural daylight in a principal component analysis (PCA)-like manner to yield primary achromatic and spectrally-opponent axes, respectively. Next, blue-cones are tuned to capture most remaining variance when opposed to green-cones. Finally, UV-cones present a UV-achromatic axis for prey capture. We note that fruit flies use essentially the same strategy to extract spectral information from their relatively blue-shifted terrestrial visual world. Together, our results suggest that rotating colour space into primary achromatic and chromatic axes at the eye’s first synapse may be a fundamental principle of colour vision when using more than two spectrally well-separated photoreceptor types.

Introduction

In visual scenes, information about wavelength is fundamentally entwined with information about intensity because the spectrum of natural light is highly correlated (Buchsbaum & Gottschalk, 1983, Chiao et al, 2000, Lewis & Zhaoping, 2006). Accordingly, wavelength information must be extracted by comparing the signals from at least two spectrally distinct photoreceptors, in a process generally referred to as “colour opponency” (Baden & Osorio, 2019). To this end, most animal eyes use up to five spectral types of photoreceptors for daylight vision, with around four being the norm for vertebrates (reviewed in (Baden & Osorio, 2019, Baden, 2021)). However, our knowledge of how the signals from four or more spectral types of photoreceptors are harnessed at a circuit level to extract this specific chromatic information remains limited.

Increasing the diversity of available spectral photoreceptors exponentially expands the diversity of theoretically detectable spectral contrasts. However, there is a law of diminishing returns: In natural scenes, some spectral contrasts are much more abundant than others. For efficient coding (Atick & Redlich, 1992, Simoncelli & Olshausen, 2001), animal visual systems should therefore prioritise the specific contrasts that are particularly prevalent in their natural visual world.

Here, we explored how zebrafish extract wavelength and intensity information from their natural visual world. Like many surface-dwelling fish, already their larvae use the ‘full’ ancient tetrachromatic cone-photoreceptor complement comprising red-, green-, blue- and UV-cones (Meier et al, 2018). Importantly, their retinal circuits can be non-invasively monitored and manipulated in the live animal (Bollmann, 2019) to provide insights into the computation of colour in the intact circuit.

We asked three questions: (i) What is the *in vivo* spectral tuning of zebrafish cone-outputs at the synapse, (ii) what is the circuit implementation, and (iii) how does this specific tuning support efficient sampling and decomposition of natural light?

Surprisingly, we found that two of the four cone types (green and blue) are strongly opponent, while the remaining two (red and UV) are essentially non-opponent, despite feeding into the same horizontal cell network. We go on to show how this spectral tuning is anatomically and functionally implemented at the circuit-level using horizontal cells. Further, comparison of the spectral tuning of the four cone-types to the spectral statistics of natural light showed that this specific cone-tuning arrangement allows zebrafish to effectively ‘solve’ a major fraction of the basic wavelength discrimination

problem already at the first synapse of their visual system: Red-cones encode “colour-invariant” achromatic information, green-cones encode “brightness-invariant” spectral information, blue-cones provide a second chromatic axis that can be further optimised by possible opposition to green cones downstream, while UV-cones by themselves provide a secondary ‘UV-achromatic’ signal – presumably for prey capture (Yoshimatsu et al, 2020). These findings also strongly imply that ancestral vertebrate circuits for colour vision are built upon the opponent signals from green- and blue-cones, which are lost in mammals including in humans (Baden & Osorio 2019).

Finally, zebrafish are not alone in using such an efficient strategy. By linking the spectral tuning of *Drosophila melanogaster* photoreceptors (Heath et al, 2020) with hyperspectral natural imaging data (Nevala & Baden, 2019) we note that fruit flies use essentially the same strategy. However, their spectral tunings are systematically blue-shifted compared to those of zebrafish, presumably to acknowledge the relatively blue-shifted statistics of natural light in air (Zimmermann et al, 2018). Taken together, our findings highlight a potentially general circuit-level mechanism of vision whereby incoming light is decomposed into “colour” and “greyscale” components at the earliest possible site.

Results

Spectral tuning of zebrafish cones in vivo. To determine spectral tuning functions of the larval zebrafish’s four cone types (Meier et al, 2018) (red, green, blue, UV), we custom-built a hyperspectral full-field stimulator based on an earlier design (Belusic et al, 2016) (Fig S3.1a,b). A diffraction grating was used to reflect the light from 14 LEDs (peaks: 360 to 655 nm) into a collimated fibreoptic that was pointed at the live zebrafish’s eye mounted under a 2-photon (2P) microscope. To avoid spectral cross-talk with the 2P imaging system, we line-synchronised each LED’s activity with the scanner retrace (Euler et al, 2016, Zimmermann et al, 2020). Together, this arrangement permitted spectrally oversampling the much broader cone opsins (FigS3.1b,c) during *in vivo* 2P imaging in the eye. All stimuli were presented as wide-field flashes from dark.

Green and blue cones, but not red and UV cones, display strong spectral opponency. We generated four cone-type specific SyGCaMP6f lines (Fig 3.1a,b), where the calcium biosensor GCaMP6f was fused to synaptophysin (Dreosti et al, 2009) such that it localised in the presynaptic terminals of each of the four cone-photoreceptors. This allowed us to measure the spectral tuning of cones at the level of

their pre-synaptic terminals (pedicles), i.e. their output (Fig 3.1c,d). Here, cones connect with other cones via gap junctions (Raviola & Gilula, 1973), with horizontal cells (HCs) which provide both feedback and feedforward inhibition (Thoreson & Mangel, 2012), as well as with bipolar cells (BCs) which carry the photoreceptor signal to the feature extracting circuits of the inner retina (Euler et al, 2014). We did not study rods, as these are functionally immature in zebrafish larvae (Branchek, 1984, Bilotta et al, 2001).

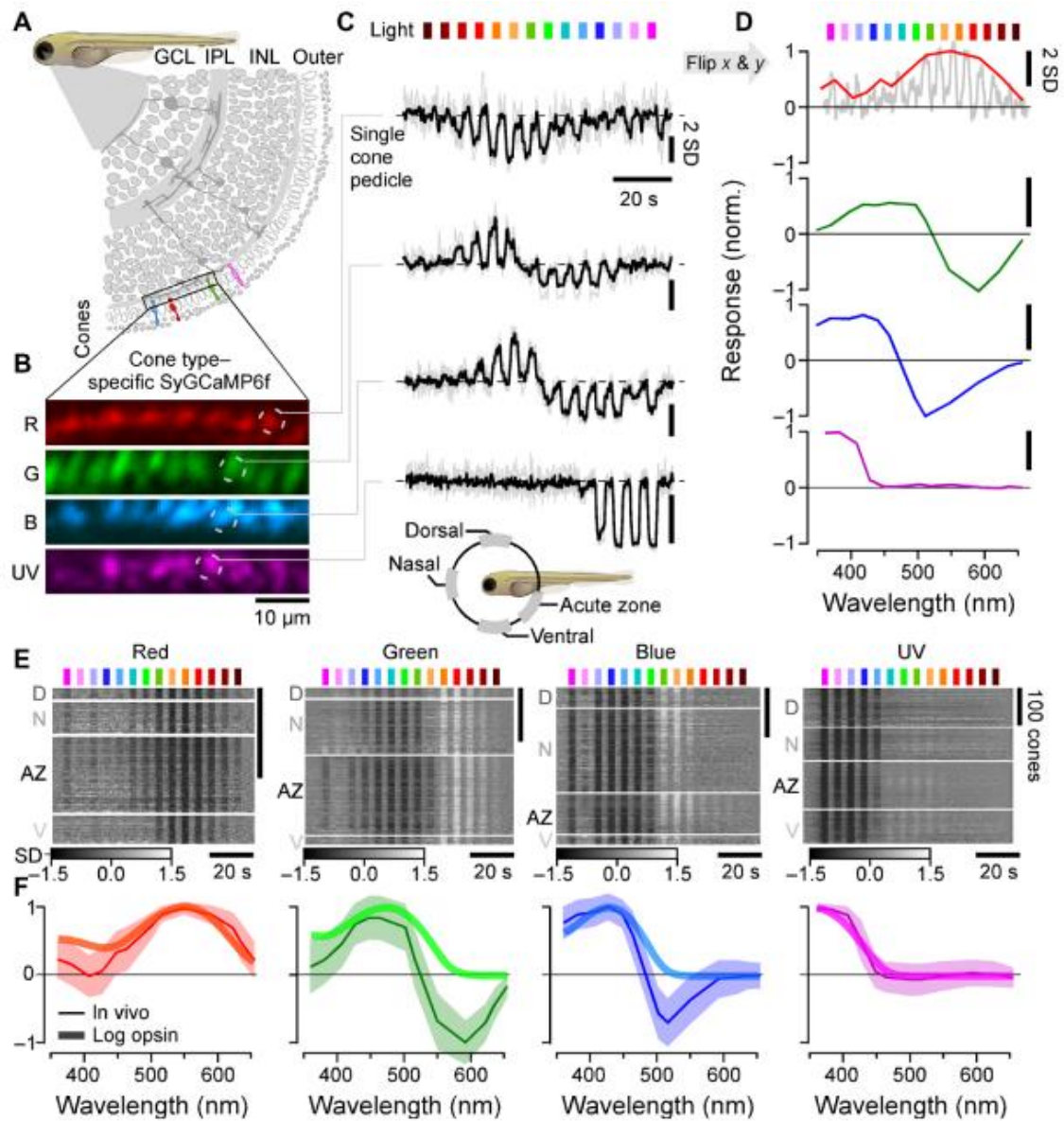


Fig 3.1: In vivo spectral tuning of larval zebrafish cones and HC block.

a, Schematic of larva zebrafish retina, with position of cone-pedicles highlighted (adapted from (82)). **b,c**, example scans of the four spectral cones (**b**, Methods) with single pedicle response examples for each (**c**) to 3 s flashes of light from each of the 14 LEDs (see Fig S3.1a-c). Shown are the means superimposed on individual repeats. **d**,

Example spectral responses summarised from (c) – note that in this representation, both the X and y axes are flipped relative to the raw responses. e,f, Population responses of each cone type recorded in different parts of the eye (D, Dorsal; N, Nasal; AZ, Acute Zone; V, Ventral – see schematic inset above for anatomical reference; vertical scalebars indicate $n = 100$ cones – see also Fig S3.1g) (e) and population mean $\pm 95\%$ confidence intervals with log-transformed respective opsin template superimposed (f, Methods). Heatmaps (e) are time-inverted to facilitate comparison to summary plots (f), greyscale bars are in z-scores. Darker shades indicate a drop in calcium relative to baseline, indicative of a cone’s “intrinsic” light response, while lighter shades indicate a rise in calcium, indicative of sign-inverted inputs from the outer retinal network – see Fig 3.2.

From fluorescence traces, we extracted tuning functions (Methods), inverting both the x- and y-axes (Fig 3.1d and inset). The inversions were done to display tuning functions from short- to long-wavelengths as is conventional, and to compensate for the fact that vertebrate photoreceptors hyperpolarise in response to light (Arshavsky et al, 2002). We adhered to the time-inversion henceforth to facilitate comparison between raw data and summary plots (e.g. Fig 3.1e). We systematically measured tuning functions for $n = 409, 394, 425, 431$ individual red-, green-, blue- and UV-cones, respectively ($n = 9, 11, 12, 7$ fish). A total of $n = 172, 288, 312, 410$ recordings, respectively, passed a quality criterion (Methods, Fig S3.1d-g) and were kept for further analysis.

Because the larval zebrafish eye is both structurally and functionally asymmetrical (Yoshimatsu et al, 2020, Zimmermann et al, 2018, Zhou et al, 2020, Schmitt & Dowling, 1999, Kolsch et al, 2020, Schroder et al, 2021), we always sampled from four different regions of the eye’s sagittal plane: dorsal (D), nasal (N), ventral (V) and the area *temporalis* (acute zone, AZ (also known as “strike zone” (Zimmermann et al, 2018))). With exceptions noted below (see also Discussion), we found that the spectral tuning of cones was approximately eye-position invariant (Fig S3.1g). For further analysis we therefore averaged across cones irrespective their position in the eye (Fig 3.1e,f).

On average, red- and UV-cones had approximately monophasic (non-opponent) output tuning functions that were largely in line with the tuning function of their respective log-transformed opsins (Methods). Such a log-transform is expected from the nature of signal transfer between outer segment phototransduction to synaptic calcium in the pedicle (Heath et al, 2020, Schneeweis & Schnapf, 1995, Schnapf et al, 1990). Red-cones were broadly tuned and never exhibited opponency (Fig 3.1f, left). In fact, some

individual red-cones hyperpolarised in response to all tested wavelengths (Fig 3.1e, left, cf. Fig S3.1g). Nevertheless, on average red-cone sensitivity was weakly suppressed in the UV-range compared to the log-transformed opsin template (Discussion). In contrast, all UV-cones were narrowly tuned up to the short-wavelength cut-off imposed by the eye optics (~350 nm, *unpublished observations*). Their tuning curve near perfectly matched the respective opsin template (Fig 3.2f, right). UV-cones in the AZ and ventral retina exhibited weak but significant opponency to mid-wavelengths (Fig S3.1g, Discussion).

Unlike red- and UV-cones, the *in vivo* output tuning functions of green- and blue-cones did not match their log-transformed opsin templates. Instead, these cones consistently exhibited strong spectral opponency to mid- and/or long-wavelength light (Fig 3.1e,f, middle). Here, blue-cones had a highly consistent zero-crossing at 483 ± 1 nm, while most green cones inverted at 523 ± 1 nm (mean, 95% confidence intervals, Methods). Green-cones in the acute zone were slightly long-wavelength shifted with a zero-crossing at 533 ± 1 nm (Fig S3.1g, Discussion).

To our knowledge, these are the first direct *in vivo* measurements of cone-pedicles' spectral tuning functions in a vertebrate.

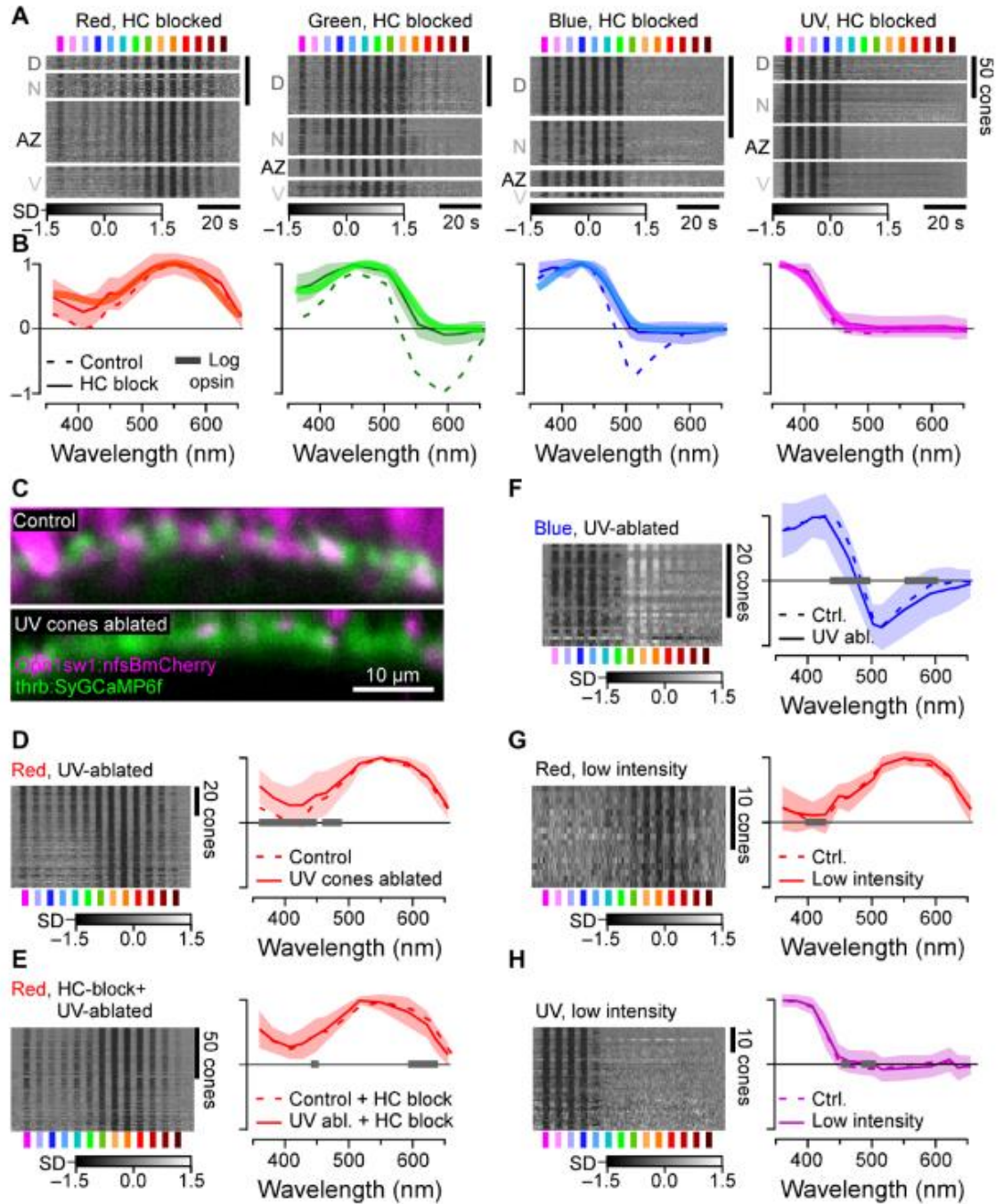


Fig 3.2: Opsin-like cone-responses in the absence of horizontal cells.

a,b, Population responses of each cone type during pharmacological blockage of HCs (**a**, Methods) and population mean $\pm 95\%$ confidence intervals with log-transformed respective opsin template superimposed (**b**, Methods). **c**, pharmaco-genetic UV-cone ablation in the background of red-cone GCaMP labelling before (top) and 24h after 2h treatment of metronidazole (10 mM) application (bottom, Methods). **d**, **e**, red-cone tunings after UV-cone ablation ($n = 77$) (**d**) and after additional pharmacological HC blockage ($n = 103$) (**e**). Shown are heatmaps (left) and means $\pm$ SD (solid lines+shadings), and analogous data in the presence of UV-cones (dotted, from Figs

3.1f, 3.2b). Note that the 361 nm LED was omitted in this experiment. **f**, as (d), but here recording from blue cones ($n = 30$). **g,h**, red- ($n = 17$) (g) and UV-cone tunings ($n = 43$) (h) at ~ 9 -fold reduced overall stimulus-light intensities (solid lines + shadings, Methods), compared to tunings at 'standard' light intensities (from Fig 3.1f). Grey bars on the x-axis in (d-h) indicate significant differences based on the 99% confidence intervals of the fitted GAMs (Methods). Note that heatmaps (a,d-h) are time-inverted to facilitate comparison to summary plots (b, d-h). Grey-scale bars in z-scores.

Spectral tuning of zebrafish cones is fully accounted for by expressed opsins and horizontal cell feedback. The nature of phototransduction in cone-photoreceptors dictates that the absorption of photons leads to a drop in synaptic calcium. Accordingly, light-driven increases in synaptic calcium (Fig 3.1f) must come from a sign-inverting connection from other cones, most likely via horizontal cells (HCs) (Klaassen et al, 2016, Chapot et al, 2017). We therefore decoupled HCs by pharmacologically blocking the glutamate output from cones using CNQX (Methods, Fig 3.2a,b). This completely abolished all spectral opponency and increased the UV-response amplitude of red cones. As a result, now all four cone-tuning functions were fully accounted for by the respective log-transformed opsins (Fig 3.2a,b, Fig S3.2a). Our results further implied that heterotypical cone-cone gap junctions, if present, do not strongly contribute to spectral cone-tuning. In support, cone-tunings were essentially invariant to additional genetic ablation of UV-cones in the absence of HCs (Fig 3.2c-f). Moreover, reducing overall stimulus brightness to probe for possible response saturation had no major effects on tuning functions (Fig 3.2g,h). Taken together, our results strongly suggest that *in vivo*, the spectral tuning of all zebrafish cones is driven by the expressed opsin variant and shaped only by specific connections with HCs relaying feedforward signals from other cones. What are these HC connections?

A connectome of the larval zebrafish outer retina. Light-microscopy studies in adult zebrafish have described at least three types of cone-HCs (H1-3), which contact R/G/B/(U), G/B/U and B/U cones, respectively (Klaassen et al, 2016, Li et al, 2009). However, for larval zebrafish HC-types and their connections to cones are not known except for H3 (Yoshimatsu et al, 2016). To complete this gap in knowledge we used a connectomics approach based a combination of serial-section electron microscopy (Fig 3.3) and confocal imaging (Fig S3.3, Methods). In total, we reconstructed a $70 \times 35 \times 35 \mu\text{m}$ patch of larval outer retina in the acute zone, which comprised $n = 140$ cones and $n = 16$ HCs (Fig 3.3a-d). UV- and blue-cones were identified directly in the EM-volume based on their characteristic OPL-proximal mitochondrial pockets (UV, Fig S3.3a) and somata (blue, Fig S3.3b), respectively. This allowed initially sorting cones

into three groups: UV, blue and red/green. Next, we traced each HC's dendritic tree and identified their connections to cones belonging to each of these cone-groups (Fig 3d-k, Fig S3.3c-h). Relating each HC's relative connectivity to UV-cones to their connections to red/green-cones allowed separating HCs into three groups (Fig 3.3i, Fig S3.3g), which were verified by clustering the HCs on all extracted features (Methods). These were dubbed H1, H2, and H3, based on their similarity to known adult HC types (Li et al, 2009, Connaughton & Nelson, 2010, Connaughton et al, 2004). The same classification was then further confirmed by confocal microscopy (Fig S3.3d-h). Of these, some HCs reliably contacted all red/green-cones within their dendritic field and were presumed to be H1. Other HCs systematically avoided approximately half of these cones. These were presumed to be H2s given that that this type of HC contacts green- but not red-cones. In line with confocal data (Fig S3.3), this allowed disambiguating red-cones (contacted only by H1) from green-cones (contacted by both H1 and H2). With the exception of $n = 14$ of 66 red-green cones that could not be unequivocally allocated due to their location at the edge of the volume (yellow, counted as 0.5 red, 0.5 green in Fig3.3b,d), this completed cone-type identifications.

From here, we quantified each HC groups' connections to the four cone types. This revealed that H1 contacted essentially all red-, green- and blue-cones within their dendritic fields, but imperfectly avoided UV-cones (Fig 3.3j,k). In contrast, H2 by definition never contacted red-cones, but contacted all other cones including UV cones. Finally, H3 was strongly dominated by UV-cone contacts, with a small contribution from blue-cones. H3 never contacted red- or green-cones. Together, this confirmed that essential features of adult HC connectivity are already present in larvae, and moreover contributed cone-weighting information for the three HC types. We next asked how this specific HC-connectivity matrix underpins cone-spectral tunings.

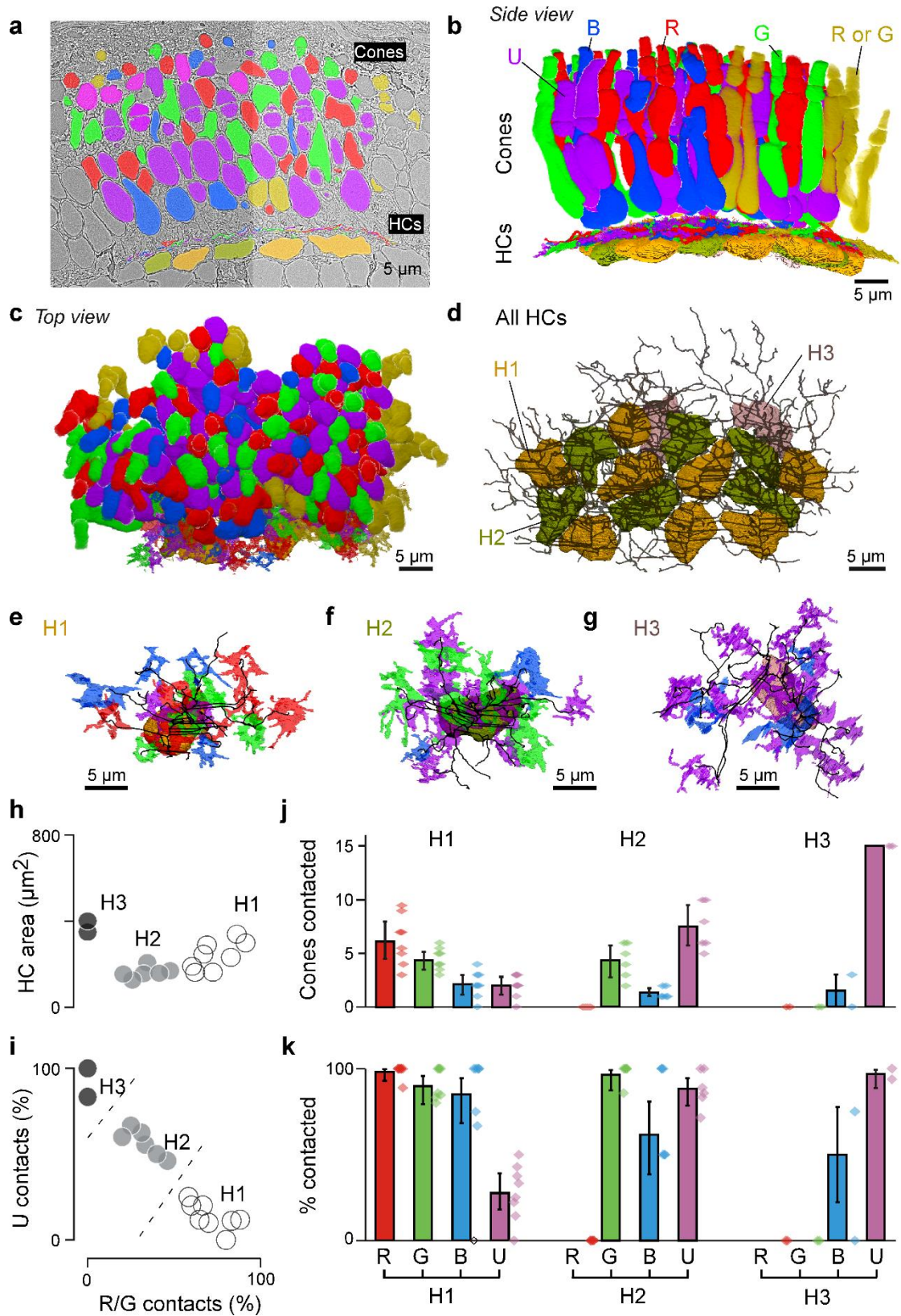


Fig 3.3: Connectomic reconstruction of outer retinal circuitry.

a, Example vertical electron microscopy (EM) section through the outer retina, with cones and horizontal cells painted. Cones are colour coded by their spectral identity, with “yellow cones” indicating red- or green-cones at the section edge that could not be

unequivocally attributed (Methods); HCs: H1, yellow/brown; H2, dark green, H3: light pink. b-d, Full volumetric reconstruction of all cones and skeletonised HCs in this patch of retina, shown from the side (b), top (c) and HC's only (d). e-g, example individual HCs classified as H1 (e), H2 (f) and H3 (g) with connecting cone pedicles. h-k, Quantification of HC dendritic area (h, cf. Fig S3.3g) and cone contacts (j-k) shown as absolute numbers with bootstrapped 95% CI (j) and percentage of cones in dendritic territory with binomial CI (i,k).

H1 horizontal cells likely underlie most spectral tuning. To explore how the three HC-types contribute to spectral cones-tunings, we first set up a series of functional circuit models for all possible combinations of HCs (Methods). These linear models included the established connectivity structure (Fig 3.3k) and were driven by the cone tunings in the absence of HCs (Fig 3.2a), with the goal of explaining cone-tunings in the presence of HCs (Fig 3.1f). We computed posteriors for the model parameters using likelihood-free inference (Lueckmann et al, 2017) based on the cones' tunings, and we assumed sign-preserving connections from cones to HCs but sign-inverting connections from HCs to cones. The model recapitulated well the *in-vivo* tuning functions of all cones when simultaneously drawing on all three HC types. However, almost the same fit quality was achieved when using H1 alone (Fig 3.4a-d, cf. Fig S3.4a-c), while H2 mainly fine-tuned the blue- and UV-cones and H3 had negligible impact on any cone-tunings (Fig S3.4a). In fact, any model that included H1 outperformed any model that excluded H1 (Fig S3.4a-c). H1, where present, also consistently provided the strongest feedback amongst HCs (Fig 3.4d, Fig S3.4c). Together, modelling therefore suggests that H1-like HCs are the main circuit element underlying the *in-vivo* spectral tuning of zebrafish cones. Moreover, the inferred relative cone-type weighting for H1 approximated their anatomical connectivity established by EM (Fig 3.4j), with the exception of green-cones which had stronger-than-expected weights (Fig 3.4d) – possibly uncovering an increased synaptic gain at this site.

Next, we sought to verify the model by experimentally measuring the spectral tunings of HCs and comparing these to the predicted HC tunings from the full model (Fig 3.4e). For this, we used *in vivo* 2P voltage imaging of HCs somata using the genetically encoded voltage biosensor ASAP3 (Villette et al, 2019) (Fig 3.4f-l). The choice of voltage over calcium imaging was motivated by a lack of detectable calcium responses in the somata of larval HCs (Methods). In total, recordings from $n = 86$ HCs that passed a quality criterion (Methods) were sorted into three clusters (Methods). The largest cluster exhibited a spectrally broad, monophasic response that closely matched the model's prediction for H1 (Fig 3.4l, see also (Klaassen et al, 2016, Connaughton &

Nelson, 2010)). Next, short-wavelength biased clusters 2 and 3 closely matched the model's prediction for H2 and H3, respectively (Klaassen et al, 2016, Connaughton & Nelson, 2010).

Efficient encoding of achromatic and chromatic contrasts in natural light. To explore how the specific *in vivo* cone tuning functions may support zebrafish vision in nature, we next computed the distribution of achromatic and chromatic content of light in their natural habitat. For this, we used a total of $n = 30$ underwater hyperspectral images (1,000 pixels each: 30,000 spectra) previously recorded in a zebrafish natural habitat in Northern India (Nevala & Baden, 2019, Zimmermann et al, 2018) (Fig 3.5a-c). Using one example scan for illustration (Fig 3.5a), we first computed each cone's view of the world in the absence of outer retinal feedback by taking the dot product of each log-transformed opsin spectrum with each pixel spectrum (Fig 3.5d-f). In this configuration, the intensity-normalised representations of the scene by each of the four cones were extremely similar as expected from high spectral correlations in natural light (Fig 3.5d). In contrast, when the same scene was computed for the intact outer retinal network by taking the *in vivo* cone-tuning functions (from Fig 3.1f), the different cones instead delivered much more distinct images (Fig 3.5g-i).

Next, to determine the spectral axes that optimally captured the variance of natural light in the zebrafish's natural underwater world (Discussion), we used principal component analysis (PCA) across the spectra of all $n = 30,000$ pixels in the data set (Fig 3.5c, j-l). Due to the strong spectral correlations in natural light, the first component (PC1) captured the achromatic ("black and white") image content, while subsequent components (PC2, PC3 etc.) captured the major chromatic ("colour") axes in decreasing order of importance (Buchsbaum & Gottschalk, 1983, Atick & Redlich, 1992). Together, PCs 1-3 accounted for 97% of the natural spectral variance (Fig 3.5m). We computed what the example scene would look like if sampled by detectors that were directly based on the first three principal components. We found that scenes processed by PC1 and PC2 (Fig 3.5j) were highly reminiscent of the scenes sampled by *in vivo* red- and green cones, respectively (Fig 3.5g). Next, PC3 was not obviously captured by either of the remaining blue- or UV-cones in isolation, however it did approximately resemble the scene when reconstructed by a green/blue-cone opponent axis ("GB", turquoise, Discussion). In fact, PC3 could be approximated by a variety of cone-combinations, however all best-matches ($\rho=0.97$, Methods) required opposing green- and blue-cones (Fig S3.5).

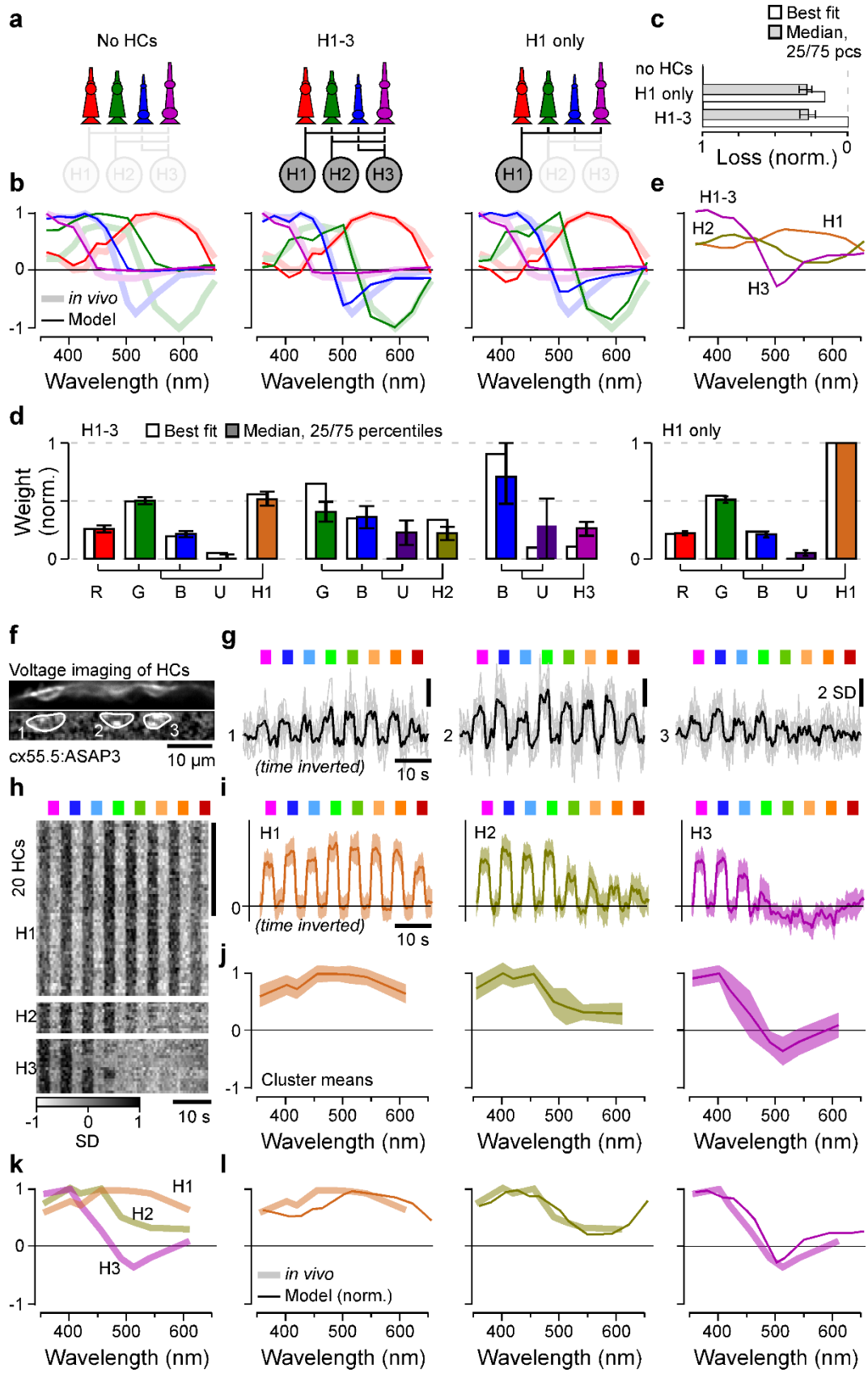


Fig 3.4: Spectral tuning of cones by horizontal cells.

a-e, linear model of spectral tuning in an outer retinal network comprised of 4 cone- and 3 HC-types, with maximum connectivity matrix defined as in Fig 3.3k (Methods). Cone tunings are initiated based on in vivo data during HC block (Fig 3.2b). Different HC combinations include (a, from left): no HCs, all HCs and H1 only. In each case, the model computes resultant cone-tunings (solid lines) superimposed on in-vivo data in the absence of HC block (shadings, from Fig 3.1f) (b), reconstruction quality (c) as loss relative to the peak performance for the full H1-3 model (loss = 0) and in the absence of HCs (loss = 1) and normalised weights such that cones contributing to a given HC, and HCs contributing to the full model, each add up to 1 (d). In addition, resultant HC tunings are shown for the full H1-3 model (e). **f-j**, in vivo voltage imaging of HC somata's spectral tuning (Methods). f,g example scan (f, average image (top) and local response correlation (83) and Regions of Interest (ROIs, bottom) and responses (g, mean superimposed on individual repeats shown for the three HC somata in this scan, of which ROIs 1 and 2 responded broadly across all tested wavelength, while ROI 3 exhibited a clear short-wavelength preference). **h-j**, results of clustering of mean responses from $n = 86$ ROIs (h, $n = 15$ fish) with cluster means (i) and extracted tuning functions (j, means $\pm$ SD). **k,l**, mean tunings of in vivo HC clusters (k, from j), and superposition of each modelled (solid lines, from e) and measured (shading, from k) HCs. Note that raw- (g) and averaged (i) HC-responses as well as the summary heatmap (h) are time-inverted to facilitate comparison with summary plots (j-l). Greyscale bar in (h) in z-scores.

Direct superposition of these cone-output spectra with the respective principal components further illustrated their striking match (Fig 3.5n). These cone-spectra were also well matched by a direct fit to the principal components when using the four cones' opsin-templates as inputs (Fig 3.5n, yellows, Methods). Here, our rationale was that these opsin-fits present a biologically plausible optimum for mimicking the principal components.

To quantitatively explore this match and its consequences for the encoding of natural light, we next computed how each of the 30,000 individual collected spectra would activate red- and green-cones as well as the GB-axis. We then plotted these activations against the respective loadings of PC1-3 for these spectra (Fig 3.6a). In each case, we also computed the same metric for the best log-opsin fits to the PCs. This confirmed the excellent performance of the system for separating achromatic from chromatic information under natural light. Red-cone activation correlated almost perfectly (mean $\rho > 0.99$, 2.5/97.5 percentiles 0.99/>0.99) with spectral loadings against PC1 (Fig 3.6a, top left, cf. Fig 3.6b, top left), but was uncorrelated with either PC2 ($\rho =$

0.16, -0.89/0.88) or PC3 ($\rho=0.29$, -0.34/0.91) (Figs 3.6a,b, middle and bottom left). Moreover, red-cone performance was near-indistinguishable from that of the opsin fit against PC1 ($\rho>0.99$, >0.99/>0.99), which was used as a biologically plausible benchmark of optimality (Figs 3.6a,b, second column). Accordingly, and despite the minor differences in short-wavelength activation of the red-cone action spectrum compared to PC1 and its opsin fit (Fig 3.5n, left, Discussion), red-cones encoded natural achromatic contrast (i.e. “brightness”, PC1) with negligible contamination of chromatic information (i.e. PCs 2,3). In contrast, activation of green-cones was highly correlated with PC2 ($\rho=0.99$, 0.98/>0.99), but uncorrelated with either PC1 ($\rho=-0.15$; -0.88/0.88) or PC3 ($\rho=0.14$, -0.66/0.81, Figs 3.6a,b columns 3). Again, their performance was near-indistinguishable from that of the respective opsin fit (Figs 3.6a,b, columns 4). Accordingly, green-cone activation carried no information about brightness, but instead encoded an efficient primary chromatic signal.

Next, both activation of the GB-opponent axis and of the corresponding opsin fit correlated strongly with PC3 ($\rho=0.95$, 0.80/0.99; $\rho=0.79$, 0.18/0.99, respectively), but not with PC1 ($\rho=0.38$, -0.25/0.92; $\rho=-0.08$, -0.76/0.52) or PC2 ($\rho=0.31$, -0.62/0.91, $\rho=-0.15$, -0.80/0.57, Figs 3.6a,b, columns 5,6). Accordingly, contrasting the signals of blue- and green-cones offers the theoretical possibility to build an efficient secondary chromatic signal in downstream circuits (Discussion). Notably, blue-cones in isolation correlated mainly with PC2 ($\rho=0.94$, 0.85/0.99) rather than PC1 ($\rho=0.18$, -0.74/0.91) or PC3 ($\rho=0.43$, -0.33/0.90) (Figs 3.6a,b, columns 7), suggesting that they could potentially serve to provide an alternative route to encoding primary chromatic information.

Finally, UV-cones mainly correlated with PC1 ($\rho=0.80$, 0.51/0.99), suggesting that this ultra-short-wavelength channel may serve to provide a secondary achromatic signal (Figs 3.6a,b, columns 8). However, its performance in doing so was substantially inferior to that of red-cones, suggesting that its primary function is not the encoding of achromatic brightness *per se*, but rather to specifically detect short-wavelength signals. Here, their weak but significant opponency to spectrally intermediate signals may serve to accentuate contrast against an otherwise “grey” background (Discussion).

Taken together, it appears that larval zebrafish effectively ‘rotate’ colour space already at their visual system’s first synapse signal along an achromatic axis (red-cones) and a primary chromatic axis (green-cones), with the added possibility to build an efficient secondary chromatic axis by opposing green- and blue-cones downstream. Together, this system captures at least 91.3% of spectral variance in natural scenes when using

red- and green- cones alone, and potentially up to 97% if including green-blue opponency. Elegantly, it also leaves UV-cones to serve independent visual functions, such as prey capture of UV-bright microorganisms (Yoshimatsu et al, 2020) (Discussion).

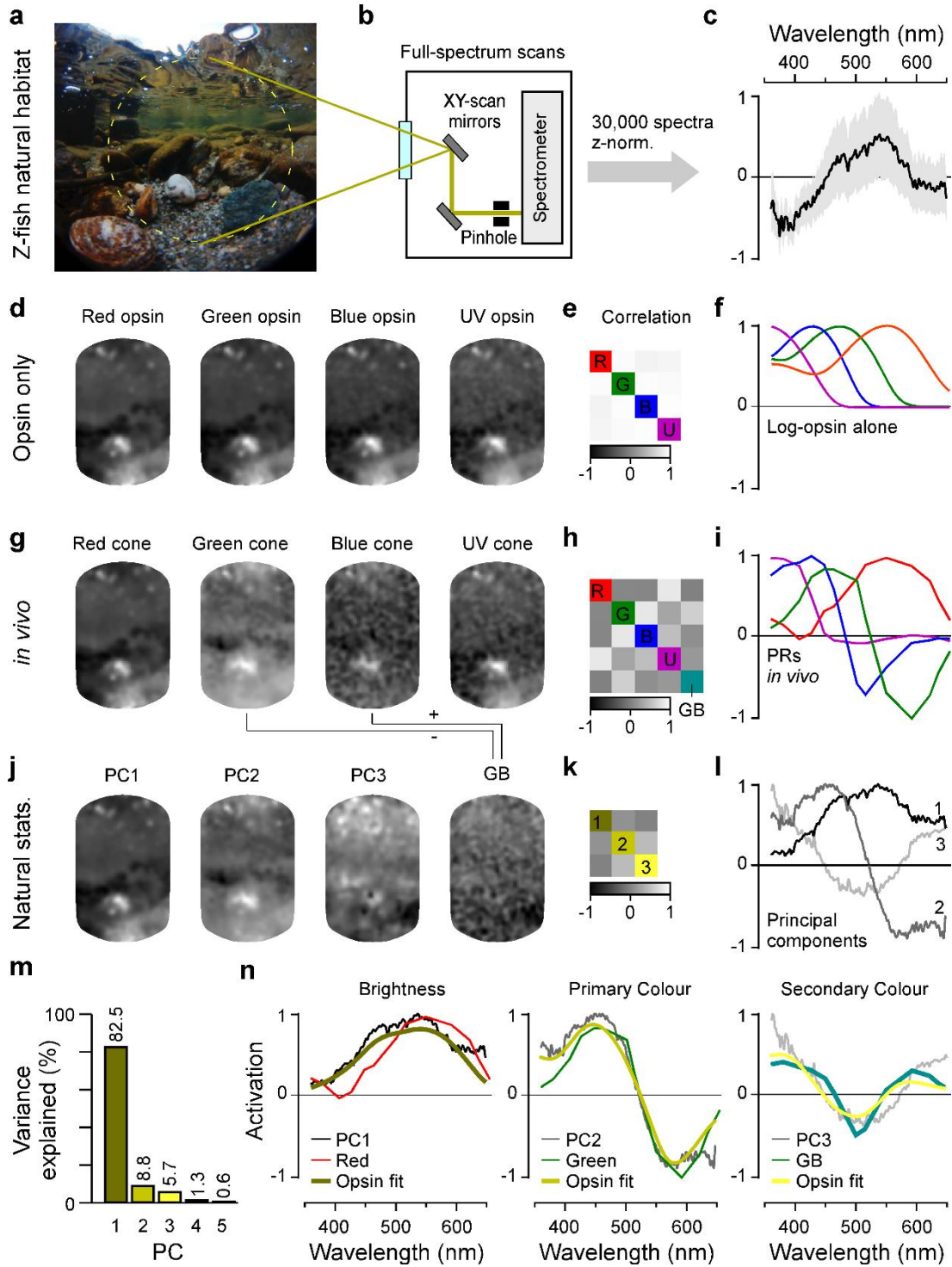


Fig 3.5: *In vivo* cone tunings efficiently represent statistics of natural light.

a-c, *Hyperspectral data acquisition from zebrafish natural visual world*. A 60° window around the visual horizon of an example scene recorded in the zebrafish natural habitat (a) was sampled at 1,000 equi-spaced points with a custom-built spectrometer-based scanner (Nevala & Baden, 2019) (b) to yield 1,000 individual spectral readings from that scene. (c) summarises the pooled and z-normalised data from $n = 30$ scenes (30,000 spectra) with $\text{mean} \pm \text{SD}$ (data from (Zimmermann et al, 2018)). Photo credit (panel a): Tom Baden, University of Sussex. **d-l**, *reconstructions and analysis of the example scene as see through different spectral filters*: (d-f) log-opsin spectra, (g-i) cone *in vivo* tunings and (j-l) based on first three principal components (PCs) that emerge from the hyperspectral data shown in (c). From left to right: (d,g,i) example scene (from a) reconstructed based on opsin-/in vivo-/PC-tunings as indicated, (e,h,k) correlation matrices between these respective reconstructions and (f,i,l) the actual tunings/PCs. A 5th element “GB” (for “green/blue”) is computed for *in vivo* tunings as contrast between green- and blue-cone tunings (cf. Fig S3.5). **m**, % variance explained by the first five principal components (l). **n**, Superposition of cone *in-vivo* tunings (coloured lines), PCs, and a linear R/G/B/U log-opsin fit to the respective PC (yellows, Methods). The latter fit can be seen as the biologically plausible optimum match to a given PC that can be achieved in a linear regime.

A comparison to spectral processing in fruit flies. A conceptually similar decomposition of natural light may also be used in *Drosophila melanogaster* (Fig 3.6c-e, Fig S3.6), the only other tetrachromatic species where *in vivo* spectral tuning functions of photoreceptor outputs are available (Heath et al, 2020). In these flies, R1-6 photoreceptors express a mid-wavelength sensitive opsin and are generally considered an achromatic channel, while R7/8-type photoreceptors are associated with colour vision (Schnaitmann et al, 2020). We therefore compared spectral tuning curves of the four varieties (yR8, yR7, pR8, pR7) of *Drosophila* R7/8-type photoreceptors (Fig 3.6c, taken from (Heath et al, 2020)) with the principal components that emerged from natural spectra of $n = 4$ daytime field and forest scenes (Nevala & Baden, 2019), each comprising 1,000 individual spectra as before (Fig 3.6d,e, Fig S3.6a-g, Discussion).

Like for zebrafish, this showed that their spectral tuning curves were well approximated by the first three terrestrial PCs: PC1 and yR8 ($p > 0.99$, $0.99 / > 0.99$), PC2 and yR7 ($p = 0.93$, $0.91 / 0.98$) and finally PC3 by opposing jointly opposing both yR8 and yR7 against pR8 (for simplicity: “yyp8”, $p = 0.72$, $0.60 / 0.84$, Fig 3.6d,e, cf. Fig S3.6d-g). Compared to zebrafish, the spectral matches between photoreceptor action spectra and natural PCs were however slightly worse, which may in part be linked to the use of a smaller natural imagery dataset, and to the comparatively lower spectral resolution

information currently available in flies. Nevertheless, this general match was made possible by the fact that, in line with the relatively increased predominance of short-wavelength light above the water (Fig S3.6a), all terrestrial principal components (Fig S3.6b) and corresponding action spectra (Fig S3.6g) were blue-shifted relative to those of aquatic environments and of zebrafish, respectively.

Together, this suggests that ‘rotating’ colour space into primary achromatic and chromatic axes (i.e. PC1-2) as early as possible, while leaving the ultra-short wavelength system largely isolated, may be a fundamental principle of colour vision when using more than two spectrally well-separated photoreceptor types, in a striking example of convergent evolution (Discussion).

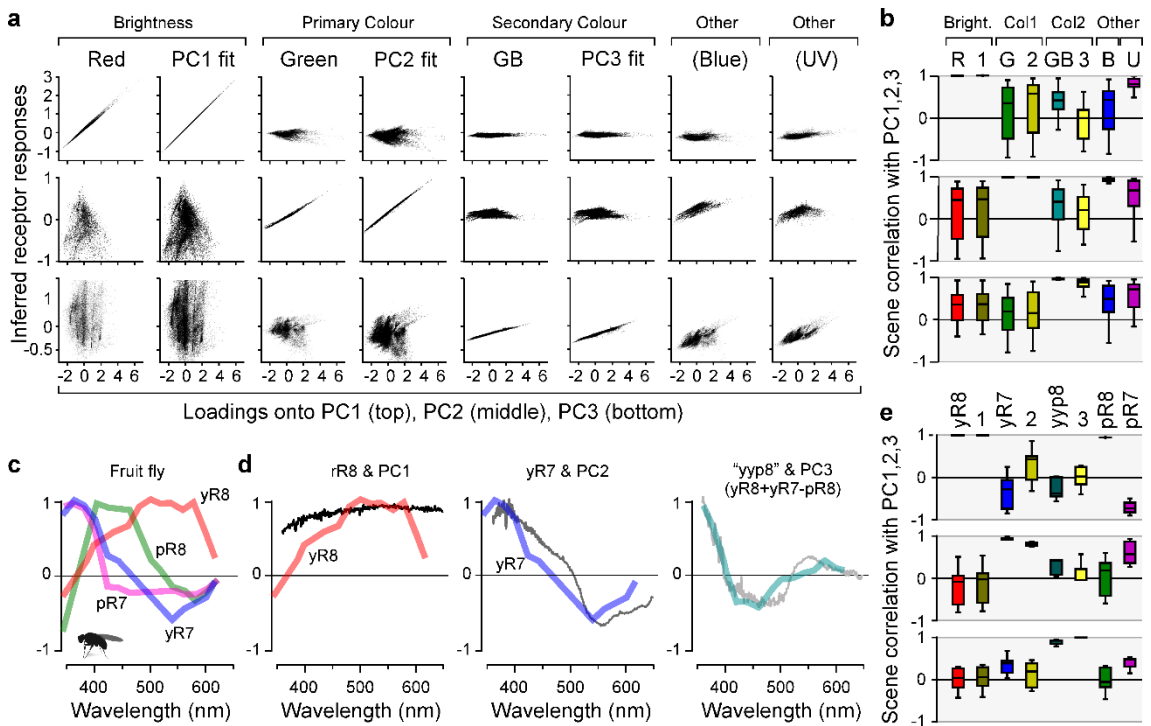


Fig 3.6: Encoding of natural achromatic and chromatic contrast.

a, Computed “responses” of *in vivo* cones, the GB-axis, and each respective log-opsin PC-fit (all from Fig 3.5i,n) to each of the $n = 30,000$ individual natural spectra, plotted against (each spectrum’s loadings onto PC1 (top row), PC2 (middle row) and PC3 (bottom row), as indicated. “Responses” plotted on y-axes, PC-loadings on x-axis. In general, a column that shows a near-perfect correlation in one row, but no correlation in both other rows (e.g. column 1) can be seen as a tuning function that efficiently captures the respective PC (e.g. column 1 shows that red-cones efficiently represent PC1 but not PC2 or PC3). **b**, Corresponding summary statistics from (a), based on scene-wise Spearman-correlations. **c**, Spectral tuning functions of *Drosophila* R7/8 photoreceptors as measured *in vivo* at their synaptic output (data from (Heath et al, 2020)). **d**, comparison of *Drosophila* tuning functions with the first three PCs that emerge from terrestrial natural scenes (data from (Nevala & Baden, 2019)). Here, PC3 is matched with a “yyp8” axis as indicated (cf. Fig S3.6d-f). **e**, Summary stats of

Drosophila photoreceptor “responses” to each of the $n = 4,000$ individual terrestrial natural spectra plotted against their respective PC loadings.

Discussion

Our physiological recordings from cones (Figs. 3.1,3.2) and horizontal cells (Fig 3.4f-l), linked to synaptic level EM-reconstructions (Fig 3.3) and computational modelling (Fig 3.4a-e) provide a comprehensive *in vivo* account of spectral processing for an efficient decomposition of natural light (Figs 3.5,3.6) at the visual system’s first synapse in a tetrachromatic vertebrate.

Linking retinal colour opponency to the principal components of natural light spectra. Using PCA of light spectra for understanding the encoding of natural scenes by animal visual systems has a long tradition, for example in information-theoretic considerations by Buchsbaum and Gottschalk in 1983. This seminal work described how the three primaries of the human eye (long- mid- and short-wavelength sensitive: L/“red”, M/“green”, S/“blue”, respectively) can be efficiently combined to derive one achromatic and two chromatic axes with none, one and two zero crossings, respectively. These theoretically optimal channels corresponded well to psychophysically determined opponent mechanisms in human vision, and were later shown to capture much of the spectral variance in natural light (Lewis & Zhaoping, 2006). However, in contrast to zebrafish, the circuit mechanisms that enable this striking link between the human primaries and perception involve multiple levels of computation across both the retina and the brain remain incompletely understood: First, many retinal ganglion cells (RGCs) and their central targets, including in visual cortices, are mid/long-wavelength-biased and non-opponent, and encode achromatic contrasts (Field et al, 2010). Second, inherited from probably non-selective retinal wiring, midlevel circuits carry “red-yellow” or “green-yellow” spectral information that is thought to be decoded into a primary “red-green” colour-opponent axis in the central brain by mechanisms that remain largely unsolved. Third, at least three types of “blue-yellow” RGCs contrast the signals from blue-cones against the sum of red- and green-cones. This RGC opponency is mainly achieved at the level of RGC dendrites, by contrasting the signals of approximately non-opponent inner retinal neurons (Dacey, 1996).

In addition, primate blue-cones themselves are yellow-blue opponent due to feed-forward inputs of red-/green-cone inputs via HCs (Packer et al, 2010) – reminiscent of the strategies employed by zebrafish cones. However, primate blue- and red-/green-cones are homologous to zebrafish UV- and red-cones, respectively (Davies et al,

2012, Musser & Arendt, 2017), and the HC underlying is H2 (Dacey, 1996). Accordingly, spectral opponency in primate blue-cones is presumably linked to the weak but significant mid-wavelength opponency of zebrafish UV-cones, rather than the much stronger opponency of zebrafish green- or and blue-cones (Fig 3.1f).

Beyond primates, comparative circuit knowledge of vertebrate retinas for spectral processing is sparse and mainly restricted to dichromatic mammals (Baden & Osorio, 2019). Amongst tetrachromats that retain ancestral green- and blue-cones, measurements of spectral responses in adult HCs of diverse species of fish (Baden, 2021, Klaassen et al, 2016, Connaughton & Nelson, 2010, Kamermans et al, 1991) are in good agreement with our *in vivo* HC data in larval zebrafish. Moreover, zebrafish inner retinal neurons (Meier et al, 2018, Zimmermann et al, 2018, Zhou et al, 2020, Wong & Dowling, 2005) display both non-opponent as well as a wide diversity of opponent responses that generally prioritise simple short-vs.-long wavelength computations over more complex combinations, broadly in agreement with predictions from theory (Buchsbaum & Gottschalk, 1983). However, in the absence of systematic and spectrally resolved sensitivity measurements of zebrafish inner retinal neurons, it has not been possible to explicitly link their properties to the variance in natural visual light. In addition, direct *in vivo* spectral measurements of zebrafish cone-photoreceptor outputs have remained outstanding.

Amongst invertebrates, *Drosophila melanogaster* stands out as the only tetrachromatic species where spectrally resolved photoreceptor output tuning functions are available (Heath et al, 2020). As discussed, these reveal a conceptual match to those of zebrafish, even down to circuit implementation involving a single horizontal-cell-like feedback neuron - all despite their eyes having evolved independently since long before the emergence of image-forming vision in any animal. Here, the authors draw on Buchsbaum and Gottschalk's ideas on efficient encoding (Buchsbaum & Gottschalk, 1983) to suggest that like for zebrafish bipolar cells (Zimmermann et al, 2018), the *Drosophila* R7/8 single and double zero-crossings can be conceptually matched with opsin-based primary and secondary colour axes, respectively. However, how this link would look like in practise for the encoding of spectral variance in natural light remained unclear. Here, we extend these theoretical links to directly show how like in zebrafish, *Drosophila* PC1 and PC2 are each well captured by a single receptor, while capturing PC3 requires possible opposing of multiple receptors downstream.

Achromatic signalling. Natural scenes are generally dominated by achromatic over chromatic contrasts (Atick & Redlich, 1992), and biased to mid- or long-wavelengths.

Accordingly, an efficient achromatic encoder should approximate the resultant mid-/long-wavelength biased mean spectrum of light in a non-opponent manner - as is the case for both zebrafish red-cones (Fig 3.2n) and for *Drosophila* yR8 photoreceptors (Fig 3.6d). Here, the quality of the spectral match primarily impacts the maximal achievable signal-to-noise of the encoder, rather than its ability to encode brightness *per se* (Vorobyev & Osorio, 1998, Bartel et al, 2021). Accordingly, despite their minor respective mismatches compared to the mean of available light (see below), both zebrafish (Fig 3.6a,b) and *Drosophila* implementations (Fig 3.6d,e) capture PC1 well. For the same reason, also other non-opponent photoreceptors, such as *Drosophila* R1-6 (Sharkey et al, 2020) as well as vertebrate rods or “true” double-cones in many non-mammalian vertebrates, are generally thought to capture achromatic signals (Baden & Osorio, 2019). However, in all these cases the presumed non-opponent nature at the level of their synaptic output *in vivo* remains to be confirmed.

In both zebrafish red-cones, and in *Drosophila* yR8, the largest mismatch to their natural environment’s PC1 was in the UV-range (Figs 3.2n, 6d). Here, it is tempting to speculate that their low short-wavelength sensitivity is linked to a need to isolate behaviourally critical “general” achromatic signals from those that incur specifically in the UV-range. In the case of zebrafish, UV-specific signals carry key visuo-ecological relevance, in that they can report the presence of prey (Yoshimatsu et al, 2020) – a rare feature that is unlikely to be captured in our scene-wide data of natural spectra (see also discussion on UV-signalling below).

Ultimately, the signals from red-cones must be read out by downstream circuits, in a manner that approximately preserves their spectral tuning. This could principally occur via a private-channel, as potentially provided by mixed-bipolar cells which in adults receive direct inputs only from red-cones and from rods (Li et al, 2012). However, most zebrafish bipolar cells receive direct inputs from more than one cone type, presumably mixing their spectral signals. Nevertheless, a PC1-like signal does filter all the way to the brain where it forms the dominant Off-response (Bartel et al, 2021).

Primary chromatic signalling. In natural scenes, all spectral variance that is not captured by PC1 is chromatic, with any subsequent components capturing progressively smaller fractions of the remaining variance in a mutually orthogonal manner. Accordingly, PC2 and PC3 are maximally informative about primary and secondary spectral contrasts, respectively, while at the same time being uninformative both about brightness (i.e. PC1), or about each other. Here, we found that zebrafish green-cones (Fig 3.2n), as well as *Drosophila* yR7 photoreceptors, both provide a good

match to their respective environment's PC2 (Fig 3.6d). In the case of zebrafish, this match was close to perfect: When challenged with natural spectra, green-cones were highly informative about PC2, but uninformative about PC1 or PC3. Accordingly, like for red-cones (discussed above), the visual system would be well-served to read out the signal from green-cones in a private-line at least once so as to preserve this already efficient chromatic signal. Indeed, green-cones are anatomically the only cones in the zebrafish retina known to have such an arrangement: two of the more than twenty zebrafish bipolar cell "morpho-types", both stratifying in the traditional "Off-stratum" of the inner plexiform layer (IPL), make exclusive contacts to green cones (Li et al, 2012). Potentially in agreement, we previously identified a small but well-defined population of singly colour-opponent bipolar cell responses in this part of the IPL (Zimmermann et al, 2018).

Further chromatic signalling. Beyond PCs 1 and 2, most of the remaining spectral variance was captured by PC3, which presents a triphasic spectral response with two zero crossings. However, neither of the remaining blue- and UV-cones exhibited such a tuning. Of these, blue- but not UV-cones were strongly opponent, nevertheless suggesting their important role in spectral processing. Accordingly, we explored why blue-cones did not directly capture PC3. For this, we returned to our horizontal cell model, this time immediately optimising red- green- and blue-cones to match PC1, PC2 and PC3, respectively. To complete the model, UV-optimisation was left unchanged to again target its own *in vivo* tuning function. Using this strategy, it was possible to produce only weakly distorted red-, green- and UV-cone spectra. However, the model failed to directly capture PC3 using blue-cones, and the mild relative distortion of green-cone spectral tuning was sufficient to noticeably degrade their ability to capture PC2 (Fig S3.6h-k). This tentatively suggests that the specific connectivity of the outer retina, constrained by the four principal zebrafish cone-opsins, is poorly suited to additionally produce a PC3-like spectral response.

Nevertheless, blue-cones did exhibit a single zero crossing that differed from that of green-cones, meaning that two zero crossing could be readily achieved in a linear model that opposed green- and blue-cone signals (Fig S3.5). We showed that such an arrangement would at least in theory allow building a spectral filter which closely captures PC3 while producing only poorly correlated responses to PC1 and PC2. Intriguingly, such a PC3-like filter is in fact observed at the level of the brain, which mainly opposes UV- and Red- "On" signals with spectrally intermediate blue/green "Off" signals (48). However, how this brain response is set-up at the level of the retina, remains unclear. Finally, a PC3-like signal could also be achieved in *Drosophila* by

opposing their two mid-wavelength sensitive yR7 and pR8 photoreceptors, however in this case the best match was achieved when in addition recruiting the more broadly tuned yR8 alongside yR7 (Fig S3.6d,f).

A private channel for detecting UV-signals? Remarkably, unlike red- green- or blue-cones, the final output of zebrafish UV-cones appeared to not be central to support dominant achromatic nor chromatic processing. In fact, UV-cones also use a nearly UV-exclusive horizontal cell (H3, Figs 3.3,3.4) (31, 34), likely for temporal tuning (11), while barely contributing to the signals of H1 and H2 (Fig 3.4d). Accordingly, outer retinal UV-circuits appear to approximately signal in isolation from those of the remaining cones. Similarly, direct contributions from the UV-sensitive pR7 photoreceptors were also not required to approximate the first three PCs that emerge from the natural spectral world of *Drosophila* (Fig 3.6d,e). In both cases, these photoreceptors contrasted their strong, short-wavelength exclusive response with weaker opposition at most other wavelengths. From here, it is tempting to speculate that these UV-systems may serve to *detect*, rather than necessarily to *spectrally contrast*, the presence of strongly UV-biased objects against a “naturally-grey” background. Such a detector would be invaluable for reporting the presence of the UV-bright single-celled microorganisms when illuminated by the sun, which larval zebrafish feed on (Yoshimatsu et al, 2020). To our knowledge, a similarly specific visuo-ecological purpose of UV-vision in *Drosophila* remains unknown. More generally, UV-light can be highly informative about edges in space, as it tends to accentuate objects’ silhouettes against bright backgrounds (Tedore & Nilsson, 2019, Cronin & Bok, 2016, Qiu et al, 2021).

In zebrafish, previous work has highlighted a key role of UV-vision across the retina and brain leading to behaviour (Yoshimatsu et al, 2020, Zimmermann et al, 2018, Zhou et al, 2020, Bartel et al, 2021, Novales Flamarique, 2016, Novales Flamarique, 2012). Most notably, the retina’s acute zone (Schmitt & Dowling, 1999) is dominated by UV-sensitive circuits (Zimmermann et al, 2018). Here, most bipolar cell terminals respond primarily to UV-stimulation, and only some in addition respond to other wavelengths (Zimmermann et al, 2018) – a general pattern that is recapitulated also at the level of the retinal ganglion cells (Zhou et al, 2020) to drive a strong UV-response in the brain (Bartel et al, 2021, Guggiana et al, 2021, Fornetto et al, 2020) which filters all the way to spinal circuits (Fornetto et al, 2020, Janiak et al, 2019). Nevertheless, despite this profound functional dominance, no anatomical study has reported the presence of UV-cone-dedicated bipolar cells, as for example in the case of green-cones (Li et al, 2012) (see above). While it remains unknown if such connectivity specifically exists in the

acute zone, it seems clear that more broadly across the retina, the signals from UV-cones are mixed with those of other cones. How this connectivity serves to support the diverse visuo-ecological needs of zebrafish UV-vision will be important to address in the future.

Regional differences in cone spectral tuning. Unlike many other aspects of larval zebrafish retinal structure (Zimmermann et al, 2018, Zhou et al, 2020, Shmitt & Dowling, 1999, Kolsch et al, 2020, Robles et al, 2014) and function (Yoshimatsu et al, 2020, Zimmermann et al, 2018, Zhou et al, 2020), the spectral tuning of zebrafish cones was remarkably eye-region invariant (Fig S3.1g). Nevertheless, small but significant regional variations were observed in all cone-types. Of these, the most striking differences occurred in red- and green-, and to a smaller extent also in UV-cones. Red-cones, and to a weaker extent also other cones, exhibited relatively narrowed tuning ventrally, and broadened tunings dorsally. These differences might help keeping cones within operational range despite the large difference in absolute amount light driving them: bright direct skylight versus dimmer reflected light from below, respectively. Next, amongst green-cones, the acute-zone exhibited the strongest short-wavelength response, resulting in a long-wavelength shift in their zero crossing. This finding is conceptually in line with an increase in absolute light sensitivity amongst UV-cones in this part of the eye (Yoshimatsu et al, 2020), however a possible visuo-ecological purpose of this shift remains to be established. Finally, mid-wavelength opponency amongst UV-cones was strongest in the AZ and ventrally, which may be linked to the behavioural need to contrast UV-bright prey against a spectrally intermediate but bright background in the upper-frontal parts of visual space (Yoshimatsu et al, 2020, Mearns et al, 2020). In contrast, larval zebrafish rarely pursue prey below or behind them (Mearns et al, 2020, Bianco et al, 2011), as surveyed by dorsal and nasal UV-cones, respectively.

How might these small but significant regional differences in spectral tuning be brought about? One possibility may relate to expressed opsin-variants: Unlike blue- and UV-cones, which each express a single opsin variant, larval zebrafish red- and green-cones can express up to two (red) or four (green) variants with slightly shifted absorption spectra (Chinen et al, 2003). Amongst red-cones, the dominant LWS-2 variant ($\lambda_{\max} = 548$ nm) is supplemented by LWS-1 expression ($\lambda_{\max} = 558$ nm) at the retina's ventral pole (Takechi & Kawamura, 2005), which may part-explain the ventral long-wavelength shift observed at the level of function (Fig S3.1g) – however it cannot explain the general “narrowing” of the red-cone response in this part of the eye. Amongst green-cones, RH2-1 ($\lambda_{\max} = 467$ nm) is dominant across the retina but

supplemented nasally by the relatively long-wavelength shifted RH2-3 ($\lambda_{\max} = 488 \text{ nm}$) and RH2-4 ($\lambda_{\max} = 505 \text{ nm}$) (Chinen et al, 2003, Takechi & Kawamura, 2005) – however no corresponding long-wavelength shift of the nasal green-cone response was noted in our physiological recordings (Fig S3.1g). Notably, the regional bias across opsin-variants becomes more pronounced with age (Takechi & Kawamura, 2005), and it is possible that any corresponding functional effects were too subtle to reliably detect in the larval stage. Instead, we wondered how eye-region differences in cone-tunings might be achieved by leveraging outer retinal circuits. To explore this, we again returned to our horizontal cell model, this time fitting it individually to only the subsets of recordings from each of the four regions. This revealed that the same anatomically established maximal connectivity matrix (Figs. 3.3,3.4) served well to produce any of these regional differences by minimally shifting their relative weights (Table S3.1). Accordingly, it seems likely that the same principal horizontal cell network produces these regional variations in tuning based on minor rebalancing of its relative input strengths.

Methods

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tom Baden (t.baden@sussex.ac.uk).

Experimental Model and Subject Details

Animals. All procedures were performed in accordance with the UK Animals (Scientific Procedures) act 1986 and approved by the animal welfare committee of the University of Sussex. For all experiments, we used 7-8 days post fertilization (*dpf*) zebrafish (*Danio rerio*) larvae. The following previously published transgenic lines were used: *Tg(opn1sw1:nfsBmCherry)* (Yoshimatsu et al, 2016), *Tg(opn1sw1:GFP)* (Takechi et al, 2003), *Tg(opn1sw2:mCherry)* (Salbreux et al, 2012), *Tg(thrb:Tomato)* (Suzuki et al, 2013). In addition, *Tg(opn1sw2:SyGCaMP6f)*, and *Tg(LCRhsp70l:SyGCaMP6f)*, *Tg(thrb:SyGCaMP6f)*, lines were generated by injecting pBH-opn1sw2-SyGCaMP6f-pA, pBH-LCRhsp70l-SyGCaMP6f-pA, or pBH-thrb-SyGCaMP6f-pA. *Tg(cx55.5:nlsTrpR,tUAS:ASAP3)*, line was generated by co-injecting pBH-cx55.5-nlsTrpR-pA and pBH-tUAS-ASAP3-pA plasmids into single-cell stage eggs. Injected fish were out-crossed with wild-type fish to screen for founders. Positive progenies were raised to establish transgenic lines.

All plasmids were made using the Gateway system (ThermoFisher, 12538120) with combinations of entry and destination plasmids as follows: pBH-opn1sw2-SyGCaMP6f-pA: pBH (Yoshimatsu et al, 2016) and p5E-opn1sw2 (Yoshimatsu et al, 2016), pME-SyGCaMP6f (Yoshimatsu et al, 2020), p3E-pA (Kwan et al, 2007); pBH-LCRhsp70l-SyGCaMP6f-pA: pBH and p5E-LCRhsp70l, pME-SyGCaMP6f, p3E-pA; pBH-thrb-SyGCaMP6f-pA: pBH and p5E-1.8thrb (Suzuki et al, 2013), pME-SyGCaMP6f, p3E-3.2thrb (Suzuki et al, 2013); pBH-tUAS-ASAP3-pA: pBH and p5E-tUAS (Suli et al, 2014), pME-ASAP3, p3E-pA. Plasmid p5E-LCRhsp70l was generated by inserting a polymerase chain reaction (PCR)-amplified Locus Control Region (LCR) for green opsins (RH2-1 to 2-4) (Tsujimura et al, 2007) into pME plasmid and subsequently inserting a PCR amplified zebrafish 0.6 kb hsp70l gene promoter region (Halloran et al, 2000) downstream of LCR. pME-ASAP3 was made by inserting a PCR amplified ASAP2s fragment (Chamberland et al, 2017) and subsequently introducing L146G, S147T, N149R, S150G and H151D mutations (Villette, 2019) in pME plasmid.

Animals were housed under a standard 14:10 day/night rhythm and fed three times a day. Animals were grown in 0.1 mM 1-phenyl-2-thiourea (PTU, Sigma, P7629) from 1 *dpf* to prevent melanogenesis. PTU has been linked with autophagy (Chen et al, 2021) and decreases tyroxine (Whittaker, 1966, Elsalini & Rohr, 2003) which is implicated in the development of cone function (Deveau et al, 2020). To minimise these possible adverse effects, care was taken to use the lowest concentration of PTU sufficient to prevent skin pigmentation (Elsalini & Rohr, 2003). For 2-photon *in-vivo* imaging, zebrafish larvae were immobilised in 2% low melting point agarose (Fisher Scientific, BP1360-100), placed on a glass coverslip and submerged in fish water. Eye movements were prevented by injection of α-bungarotoxin (1 nL of 2 mg/ml; Tocris, Cat: 2133) into the ocular muscles behind the eye. For some experiments, CNQX (~0.5 pl, 2 mM, Tocris, Cat: 1045) or meclofenamic acid sodium salt (MFA) (~0.5 pl, 5 mM, Sigma, Cat: M4531) in artificial cerebro-spinal fluid (aCSF) was injected into the eye.

Method Details

Light Stimulation. With fish mounted on their side with one eye facing upwards towards the objective, light stimulation was delivered as full-field flashes from a spectrally broad liquid waveguide with a low NA (0.59, 77555 Newport), positioned next to the objective at ~45°. To image different regions in the eye, the fish was rotated each time to best illuminate the relevant patch of photoreceptors given this stimulator-geometry. The other end of the waveguide was positioned behind a collimator-focussing lens complex (Thorlabs, ACL25416U-A, LD4103) which collected the light

from a diffraction grating that was illuminated by 14 spectrally distinct light-emitting diodes (LEDs) (details on LEDs below). Following the earlier design (Belusic, 2016), the specific wavelength and relative angle of each LED to the diffraction grating defined the spectrum of light collected by the collimator to be ultimately delivered to the fish's eye, according to:

$$\alpha(\lambda) = \sin^{-1}(G\lambda - \sin\beta)$$

where α is the angle of light incident to the diffraction grating, λ the wavelength (in nm), β the first order diffraction exit angle and G the diffraction grating's groove density. Moreover, each LED was individually collimated (Signal Construct SML 1089 - LT-0454) and attached to a rail (Thorlabs, XE25L450/M; XE25L225/M) by a 3D printed holder (available at <https://github.com/BadenLab/HyperspectralStimulator>).

An Arduino Due (Arduino) and LED driver (Adafruit TLC5947) were used to control and drive the LEDs, respectively. Each LED could be individually controlled, with brightness defined via 12-bit depth pulse-width-modulation (PWM). To time-separate scanning and stimulating epochs, a global "blanking" signal was used to switch off all LEDs during 2P scanning but enable them during the retrace, at line-rate of 500 Hz (see also (Euler et al, 2019, Zimmermann et al, 2020)). The stimulator code is available at <https://github.com/BadenLab/HyperspectralStimulator>.

LEDs used were: Multicomp Pro: MCL053RHC, Newark: C503B-RAN-CZ0C0AA1, Roithner: B5-435-30S, Broadcom: HLMP-EL1G-130DD, Roithner: LED-545-01, TT Electronics: OVLGC0C6B9, Roithner: LED-490-06, Newark: SSL-LX5093USBC, Roithner: LED450-03, VL430-5-1, LED405-03V, VL380-5-15, XSL-360-5E. Effective LED peak spectra as measured at the sample plane were, respectively (in nm): 655, 635, 622, 592, 550, 516, 501, 464, 448, 427, 407, 381, 361, 360 nm. Their maximal power outputs were, respectively (in μW): 1.31, 1.06, 0.96, 0.62, 1.26, 3.43, 1.47, 0.44, 3.67, 0.91, 0.24, 0.23, 0.04, 0.20. From here, the first ten LEDs (655 – 427 nm) were adjusted to 0.44 μW , while the four UV-range LEDs were set to a reduced power of 0.2 μW (407, 381, 360 nm) or 0.04 μW (361 nm). This relative power reduction in the UV-range was used as a compromise between presenting similar power stimulation across all LEDs, while at the same time ameliorating response-saturation in the UV-range as a result of the UV-cones' disproportionately high light sensitivity (Yoshimatsu et al, 2020, Zhou et al, 2020). In this regard, we took advantage of the strong spectral overlap between the two shortest-wavelength LEDs (360, 361 nm) to probe this wavelength range at two intensities (0.2 and 0.04 μW , respectively).

From here, all spectral tuning functions were based on the responses to the 13 spectrally distinct LEDs, excluding the response to low-power 361 nm LED. This strategy yielded biologically highly plausible spectral sensitivity functions in all cones that closely resembled their underlying opsin's tuning when pharmacologically isolated from horizontal cells (Fig 3.2b). Nevertheless, UV-cones weakly but consistently under-shot their opsin template at the shortest tested wavelength (360 nm), hinting that they may have approached their saturation point at this wavelength and power. In agreement, the 0.04 μ W 361 nm LED elicited only mildly lower response-amplitudes in UV-cones compared to the 0.2 μ W 360 nm LED ($R_{\text{low}} = 0.88 \pm 0.14$; $R_{\text{high}} = 0.96 \pm 0.06$, errors in SD; difference $p \ll 0.001$ Wilcoxon signed-rank test). In contrast, all other cones responded much more weakly to the low power UV-LED: Blue-cone ($R_{\text{low}} = 0.35 \pm 0.16$; $R_{\text{high}} = 0.67 \pm 0.21$); green-cone ($R_{\text{low}} = -0.12 \pm 0.24$; $R_{\text{high}} = 0.09 \pm 0.32$); red-cone ($R_{\text{low}} = -0.02 \pm 0.27$; $R_{\text{high}} = 0.21 \pm 0.27$; all low-high pairs $p \ll 0.001$) suggesting that these cones were not near their UV-saturation points.

Together, it therefore remains possible that measured cone-tuning functions relatively underestimate UV-components, however this effect is likely to be very small in non-UV-cones that dominate “traditional” colour vision in zebrafish (Discussion). The exact slope of the cones' UV-response also had negligible impact on their relative matches with PCs or their contributions to the HC-network (not shown), in line with an only weak interdependence of the outer retina's UV- versus red-/green-/blue-cone systems (see Discussion).

2-photon calcium and voltage imaging. All 2-photon imaging was performed on a MOM-type 2-photon microscope (designed by W. Denk, MPI, Martinsried; purchased through Sutter Instruments/Science Products) equipped with a mode-locked Ti:Sapphire laser (Chameleon Vision-S, Coherent) tuned to 960 nm for SyGCaMP6f and ASAP3 imaging. To measure HC tuning functions, we first expressed GCaMP6f in HCs. However, while we observed strong light-driven calcium responses at their dendritic tips, adjacent to cone terminals and thus indicative of local processing, we did not observe robust calcium responses in the HC soma (as a proxy of global processing). This lack of somatic calcium responses could be due to a putative lack of voltage-gated calcium channels in larval HC somata (unlike e.g. in adult mouse (Chapot et al, 2017)). Instead, we therefore measured voltage responses using the genetically encoded voltage sensor, ASAP3 (Villette et al, 2019), which presumably also gave a more direct readout of HC global function. We used two fluorescence detection channels for SyGCaMP6f/ASAP3 (F48x573, AHF/Chroma) and mCherry (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 6.3 for Windows (Wavemetrics). Recording configurations were as follows: UV-cone SyGCaMP6f 128x128 pixels (2 ms per line, 3.9 Hz) or 256x256 pixels (2 ms per line, 1.95 Hz); all other cones SyGCaMP6f and horizontal cell ASAP3 256x256 pixels (2 ms per line, 1.95 Hz).

Pre-processing and extraction of response amplitudes of 2-photon data. Regions of interest (ROIs), corresponding to individual presynaptic cone terminals were defined automatically based on local thresholding of the recording stack's standard deviation (s.d., typically > 25) projection over time, followed by filtering for size and shape using custom written scripts running under IGOR Pro 6.3 (Wavemetrics), as used previously (11). Specifically, only ellipsoidal ROIs (<150% elongation) of size 2-5 μm^2 were further analyzed. For ASAP3 recordings, ROIs were manually placed to follow the shape of individual HC somata. Calcium or voltage traces for each ROI were extracted and z-normalized based on the time interval 1-6 s at the beginning of recordings prior to presentation of systematic light stimulation. A stimulus time marker embedded in the recording data served to align the traces relative to the visual stimulus with a temporal precision of 2 ms.

Following the approach used in (Baden et al, 2016), a quality criterium (QC) of how well a cell responded to a stimulus were computed as

$$QC = \frac{Var[\langle C \rangle_r]_t}{Var[\langle C \rangle_t]_r}$$

where C is the T by R response matrix (time samples by stimulus repetitions) and $\langle \rangle_x$ and $Var[\]_x$ denote the mean and variance across the indicated dimension, respectively. If all trials are identical such that the mean response is a perfect representative of the response, QC is equal to 1. If all trials are random with fixed variance, QC is equal to 1/R. For further analysis, we used only cells that responded well to the stimulus (QC > 0.4 for SyGCaMP6f or > 0.32 for ASAP3) (see also Fig S3.2b)

After filtering out poorly responsive cells using QC, outliers were removed using principal component analysis. Because in all cone types, PC1 explained >80% variance of the data, we computed the loading values of the principal component 1 of cone tuning function within each cone type and defined outliers as the cones with PC1 loading below 1.25 times the length of the 97 percentile departure from the mean.

To extract response amplitudes to each stimulus wavelength, an exponential curve was fit to the entire rising (or falling, for hyperpolarising responses) phase during each stimulus presentation, with the maximum value of the fitted curve was taken as the response amplitude. Because cones are intrinsically “Off-cells” (i.e. hyperpolarize to light) we then sign-inverted extracted amplitude values such that Off-responses would yield positive amplitude readings, and vice versa for On-responses. However, for voltage imaging, because ASAP3 fluorescence intensity increases as cells hyperpolarize, we preserved the polarity of the response amplitudes.

Immunostaining and confocal imaging. Larval zebrafish (7-8 dpf) were euthanised by tricane overdose and then fixed in 4% paraformaldehyde (PFA, Agar Scientific, AGR1026) in PBS for 30 min at room temperature. After three washes in PBS, whole eyes were enucleated and the cornea was removed by hand using the tip of a 30 G needle. Dissected and fixed samples were treated with PBS containing 0.5% TritonX-100 (Sigma, X100) for at least 10 mins and up to 1 day, followed by the addition of primary antibodies. After 3-5 days incubation at 4°C, samples were washed three times with PBS 0.5% TritonX-100 solution and treated with secondary antibodies. After one day incubation, samples were mounted in 1% agar in PBS on a cover slip and subsequently PBS was replaced with mounting media (VectaShield, H-1000) for imaging. For HC imaging (Fig S3.6c-f), the retina was flat-mounted with the photoreceptors facing to the cover slip. For cone side-view imaging (Fig S3.6a), the lens was kept attached the retina to maintain the spherical shape of the retina, with the whole “retina-ball” mounted with the lens side facing to the cover slip. All presented data was imaged in the acute zone.

Primary antibodies were zpr-1 antibody (mouse, 1:100, ZIRC). Secondary antibodies were DyLight647 anti-mouse (Donkey, 1:500, Jackson Immunoresearch Laboratories). Confocal image stacks were taken on a TSC SP8 (Leica) with a 63x oil immersion objective (HC PL APO CS2, Leica). Typical voxel size was 90 nm and 0.5 μ m in xy and z, respectively. Contrast, brightness and pseudo-colour were adjusted for display in Fiji (NIH).

To sparsely label HCs, plasmids pCx55.5:Gal4 and pUAS:MYFP were co-injected into one-cell stage eggs (Yoshimatsu et al, 2014).

UV-cone ablation. Larval zebrafish were immersed in fish water containing 10 mM Metronidazole (Met) for 2 hours to ablate nfsB-expressing UV-cones. Following Met treatment, zebrafish were transferred into fish water without Met and fed regularly until used for two-photon imaging.

Electron-microscopy data acquisition, reconstruction and annotation. A larval zebrafish (8 dpf) was euthanised by tricane overdose and then a small incision on a cornea was made using 30G needle in a fixative solution containing 4% glutaraldehyde (AGR1312, Agar Scientific,) in 0.12M cacodylate buffer, pH 7.4. The tissue was immediately transferred into a 1.5 ml tube with the fixative, centrifuged at 3,000 rpm for 3 min, and further fixed in the fixative over-night on a shaker at room temperature. Subsequently, the tissue was washed 3 times in 0.12M cacodylate buffer, pH7.4 and incubated in a solution containing 1.5% potassium ferrocyanide and 2% osmium tetroxide (OsO₄) in 0.1M cacodylate buffer (0.66% lead in 0.03M aspartic acid, pH 5.5) for 1 hour. After washing, the tissue was placed in a freshly made thiocarbohydrazide solution (0.1g TCH in 10 ml double-distilled H₂O heated to 600 C for 1 h) for 20 min at room temperature (RT). After another rinse, at RT, the tissue was incubated in 2% OsO₄ for 30 min at RT. The samples were rinsed again and stained *en bloc* in 1% uranyl acetate overnight at 40 C, washed and stained with Walton's lead aspartate for 30 min. After a final wash, the retinal pieces were dehydrated in a graded ice-cold alcohol series, and placed in propylene oxide at RT for 10 min. Finally, the sample was embedded in Durcupan resin. Semi-thin sections (0.5 -1 μ m thick) were cut and stained with toluidine blue, until the fiducial marks (box) in the GCL appeared. The block was then trimmed and mounted in a Serial-blockface scanning electron microscope (GATAN/Zeiss, 3View). Serial sections were cut at 50 nm thickness and imaged at an xy resolution of 5 nm. Two tiles, each about 40 μ m x 40 μ m with an overlap of about 10%, covering the entire photoreceptor and horizontal cell layers in a side view at the acute zone were obtained. The image stacks were concatenated and aligned using TrackEM (NIH). The HCs and cones were traced or painted using the tracing and painting tools in TrackEM2 (Cardona et al, 2012).

Clustering of HCs in EM and Confocal data. To validate the ad hoc group assignment based on UV contacts (HC area) and R/G contacts for the electron microscopy (Fig 3.3h,i) and confocal data (Fig S3.3g) we used Mixture of Gaussian (MoG) clustering on all extracted features. These features (area size, number of contacts to R/G, B, U, for EM and area size, tip density, number of contacts to R, G, B/U for CM) were z-normalized and clustered in the same framework as the HC recordings (see below). The MoG clusters did coincide with the ad hoc group assignment.

Opsin Templates and log transforms. For the log-transformed opsin templates (Fig 3.1f, 3.2b) we assumed a baseline activation (represented by b in Eq. 2) and fit a linear transformation to take the arbitrary scaling of the recordings into account. We then

optimized the function $f_{a,b,c}$ to minimize the mean squared error (MSE) between $f_{a,b,c}(opsin)$ and the data of the HC block condition for each cone type:

$$a, b, c = \underset{a,b,c}{\operatorname{argmin}} \operatorname{MSE}(f_{a,b,c}(opsin), y) \quad (1)$$

where y is the mean of the HC block condition and f is the function

$$f_{a,b,c}(x) = a \cdot \log(x + b) + c. \quad (2)$$

For the optimization we used the python package `scipy.optimize.minimize` (version 1.4.1). The inverse of this procedure is shown in Fig S3.4a, where the mean of HC block condition is fitted in the same way to the opsin curves of each cone with the function:

$$f'_{a',b',c'}(x) = a' \cdot \exp(b' \cdot x) + c' \quad (3)$$

The data distribution (25 and 50 and 75 percentiles) is then calculated by passing each individual HC block recording through the optimized function f' .

Model of cone and HC interaction. We modelled cone-HC interactions as a linear model and included the established (Fig 3.3k) connectivity pattern for the three types of HC as a (3x4) connectivity matrix W where w_{ij} indicates connection strength from cone type j to HC type i . Further, we assumed the feedback strength per connection of each HC type to be constant for all cones and defined it as a diagonal matrix A . To compute the effective feedback, this matrix is then weighted by the relative connection strength per cone and HC, represented in a (4x3) matrix F with $f_{ij} = \frac{w_{ij}}{\sum_k w_{jk}}$. This represents the strength from HC type j to cone of type i . Hereby we assume a symmetric connectivity pattern which is justified by the symmetrical cone mosaic in zebrafish. With these definitions, we can formulate the model recurrently as following:

The inputs to the HCs is defined as

$$H_{in}(\lambda) = W \cdot \kappa(\lambda),$$

where $\kappa(\lambda)$ represents the raw activity in the synapse, which still has to be shifted according to the baseline. The summed outputs of the HCs are computed as

$$H_{out}(\lambda) = F \cdot A \cdot H_{in}(\lambda),$$

Finally, the raw activity in the synapses is computed as

$$\kappa(\lambda) = o(\lambda) - H_{out}(\lambda)$$

where $o(\lambda)$ represents the wavelength dependent opsin activation.

The same formulas hold for computing the baseline of the cones, for which $o(\lambda)$ was set to **1**, which accords to the applied normalization on the recorded data. The final output of the model are the tuning curves κ shifted to the cone specific baselines and normalized.

The same normalization procedure was applied to the shown HC spectra, which are the normalized spectra $H_{in}(\lambda)$.

In the reduced models, in which we only included specific types of HCs, we set the corresponding entries in the weight matrix W to zero but did not change the model otherwise.

Model input. To extract the cone tuning curves from the experimental data for the model, we computed the mean amplitude of each bright and dark three seconds interval but excluded in each interval the first second as adaption time. We then took for every individual trace the difference of each bright interval to its preceding dark interval based on these means. Finally, we averaged over these values for each cone type and experimental condition and, by assuming smooth tuning functions, interpolated (using the scipy function `scipy.interpolate.interp1d`) the data to an equidistant resolution of 1nm.

As input to our model we took the normalized traces of the blocked HC condition. This normalization can be interpreted as a maximal dark current of 1 and a minimal current of 0 during activation. The input acted as "opsin-sensitivity" curves $o(\lambda)$ of the cones. We decided to use these curves instead of the theoretical available opsin tuning curves since we have a pure linear model and as shown in Fig 3.2b these traces are a good proxy for the log-transformed opsin templates, which is the effective activation for this linear model. All spectral tuning curves of the cones were normalized to have a maximal absolute value of one.

Fitting procedure. We used the Sequential Neural Posterior Estimation method (also called SNPE-B) described in (Lueckmann et al, 2017) (code available at <https://github.com/mackelab/delfi>, version: 0.5.1) with small modifications which were already applied in (Oesterle et al, 2020) to fit our model.

In brief, SNPE-B draws parameters $\{\theta_i\}_{i \in I}$ over several rounds $r = 1, \dots, R$ from a (proposal) prior $\tilde{p}_r(\theta)$ and evaluates the model for these parameters. For the evaluations $e_i(\theta_i)$ the discrepancy function $x_i(e_i) = D(e_i)$ is computed and a mixture density network (MDN) $q_\phi(\theta, x)$ is trained on the data pairs $\{(\theta_i, x_i)\}_{i \in I}$. The posterior $p_r(\theta|x_0)$ is then calculated as $q_\phi(\theta|x = x_0)$ and used as a new proposal prior in the next sampling round: $\tilde{p}_{r+1}(\theta) = p_r(\theta|x_0)$. We took the MSE between model output and the data as discrepancy function. This implies $x_0 = 0$, but as our data is noisy, our model cannot get to a MSE of zero. This would mean, that the MDN has to extrapolate to unreached discrepancy values, which could lead to an unstable behaviour. As a consequence, we took as x_0 the 0.01-percentile of $\{x_i\}_{i \in I}$ in each round. This evaluation of $q_\phi(\theta|x = x_0)$ can be understood as the posterior over the parameters for the "best possible" model evaluations. Testing for different percentiles in a reasonable range did not change the results. We took the same approach for setting an adaptive bandwidth for the kernel (see also (Oesterle et al, 2020)). As for a few models the posteriors became slightly worse after some rounds, we compared post-hoc the posterior distributions of each round and took the one with the smallest 1-percentile of its samples.

We ran SNPE-B over five rounds, with 200,000 samples per round. The prior was a multivariate normal distribution with mean 1_n and covariance $0.25 \cdot Id_n$, where n is the number of model parameters, ranging from 11 (all HCs) to 3 (only H2). We chose three Gaussian components for the MoG and a MDN with two hidden layers with 100 nodes each. In each round the network was trained for 600 epochs with a minimum batch size of 500 and continuous learning started in round two. To let the MDN focus on regions of low discrepancy, we used a combined Uniform-Half-Gaussian kernel which was constant 1 up to x_0 and decayed then as a half Gaussian. The scale of the Half-Gaussian part was in each round chosen as the 20-percentile of the discrepancy values. For the presented tuning curves 100,000 samples were drawn from the final posterior and the model evaluated.

HC clustering based on spectral tuning. To identify functional clusters we used a Mixture of Gaussians model (`sklearn.mixture.GaussianMixture`, version 0.21.2) with three components and diagonal covariance matrices on the pre-processed tuning curves ($n = 86$) which were additionally normalized to have maximal value of one. Aiming for a stable clustering, we ran the algorithm 1,000 times with different random seeds and chose the ones with the smallest BIC and under these chose the partition which appeared most often. The different runs did not change the general shape of the

cluster means, but the specific assignment was variable for some traces. With this procedure we got a partition with $n = 12, 19, 55$ elements, which were allocated to the known functional tunings for HCs of adult zebrafish (Klaassen et al, 2016, Connaughton & Nelson, 2010).

Natural Imaging Data Analysis. The hyperspectral data were element-wise multiplied with a deuterium light source derived correction curve [S.x]. The data were restricted to the domain of 360-650 nm and z-normalised within a given scan. Here, the long-wavelength end of the domain was decided based on the long-wavelength opsin absorption curve; the short-wavelength end was dictated by the sensitivity of the spectrometer. The hyperspectral PCs were obtained using the scikit-learn 0.22.1 implementation of the Principal Component Analysis algorithm. Only the first three components are displayed.

Hyperspectral measurement points were spatially aligned within the scan according to the scan raster (see (Nevala & Baden, 2019, Zimmermann et al, 2018) for details). Pixel brightness is the projection of a given PC, or mean of the convolution with the opsin absorption or the observed cone response curves respectively. Presented images were smoothed using a Gaussian filter ($\sigma = 2\text{px}$). Sum of Squares difference was taken between pairs of z-normalised images as well as their negatives. The lowest Sum of Squares (=highest correlation, either with the original or the negative) is displayed. Smoothing did not significantly affect this measure.

To statistically compare scene reconstructions by different sets of tuning functions (Fig S3.6a-c), we used two parallel strategies. First, we computed the correlation coefficient between reconstructions by the different channels (e.g. *in vivo* red cone vs. green cone) as indicated for each of $n = 30$ scenes, thus yielding 30 correlation coefficients for each combination of channels in each condition. Amongst each comparison we then computed the mean and SD, as shown.

Second, to capture the multivariate dependence directly, we computed the mutual information under Gaussian assumption, $MI = \sum_i h(x_i) - h(x) \sim \log \det[2 \pi e C]$, where C is the correlation matrix of the scene representations in the different channels (e.g. 4x4 *in vivo*: red-, green-, blue-, UV-cone). As the diagonal of C is constant and equal to 1, the mutual information is proportional to the latter quantity. We normalized this quantity by the mutual information of the opsin set of tuning functions.

Linking opsin- and photoreceptor-spectra to principal components. Measured *in vivo* spectra of cones and their underlying log-transformed opsin templates (Fig 3.1f)

were linearly combined to provide least-squares fits to the respective underwater spectral PCs (Fig 3.5n, 3.6d, Figs. S3.5,3.6). The same procedure was also used to match *Drosophila* R7/8 spectra (Fig 3.6c, from (Heath et al, 2020)) to the PCs that emerged from natural distribution of light above the water. Next, to compare the expected responses of *in vivo* photoreceptors, their linear combinations (in case of PC3, see below), as well as their respective log-opsin constructs to natural light, individual natural light pixel spectra ($n = 30,000$) were multiplied with the respective sensitivity curves. In each case, pixel-spectra were first z-normalised within the scene, and products were summed over all wavelengths. This procedure produced 'responses' (Fig 3.6a), which were plotted against the respective loadings of each spectrum onto PC1, PC2 and PC3 (in rows 1, 2 and 3, respectively). From here, scene-wise summary statistics were computed based on Spearman correlation coefficients (Fig 3.6b,e).

To arrive at *in vivo* photoreceptor combinations that best approximated PC3s zebrafish: Fig S3.5a-c, *Drosophila*: Fig S3.6e,f), we assessed the spectral matches to them by several plausible linear combinations of *in-vivo* photoreceptor tunings based on least squares. In both cases, the best fits required opposing the two spectrally intermediate receptors. For zebrafish, this "GB-fit" performed as well as any combination of more complex fits that in addition used red- or UV-cones, so we used this simplest GB-fit for further analysis. In case of *Drosophila*, best performance required also adding the long-wavelength sensitive receptor to yield an $yR8+yR7-pR8$ axis (short: "yyp8"). In each case, performance as shown in Figs S3.5c and Fig S3.6f (top) was evaluated based on the mean scene-wise Spearman correlation coefficient between the resultant spectral axis, as described above. The weights needed to build these PC3-like tunings based on photoreceptor types are plotted below as abs(max)-normalised for better comparison.

Quantification and Statistical Analysis

Statistics. No statistical methods were used to predetermine sample size. Owing to the exploratory nature of our study, we did not use randomization or blinding.

We used Generalized Additive Models (GAMs) to analyse the relationships between wavelength and cone activity under different experimental conditions (Fig 3.2d-h, Fig S3.2). GAMs can be understood as an extension to the generalized linear model by allowing linear predictors, which depend on smooth functions of the underlying variables (Wood, 2017). We used the mgcv-package (version 1.8-31) in R on an Ubuntu 16.04.6 LTS workstation with default parameters. We modelled the dependence of the variable of interest as a smooth term with 13 degrees of freedom.

The models explained ~59-82% of the deviance. Statistical significance for differences between the dependence of activation in the different experimental conditions were obtained using the `plot_diff` function of the `itsadug`-package for R (version 2.3). Significance of opponency (Fig S3.1g) and zero crossings of the tuning curves (Fig 3.1f, Fig S3.1g) were also calculated based on GAMs with “zone” as an additional predictive variable and grouping where applicable.

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Data and Material Availability. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Plasmids pBH-opn1sw2-SyGCaMP6f-pA, pBH-LCRhsp70l-SyGCaMP6f-pA, pBH-thrb-SyGCaMP6f-pA, pBH-cx55.5-nlsTrpR-pA, pBH-tUAS-ASAP3-pA and transgenic lines *Tg(opn1sw2:SyGCaMP6f)*, *Tg(LCRhsp70l:SyGCaMP6f)*, *Tg(thrb:SyGCaMP6f)*, *Tg(cx55.5:nlsTrpR,tUAS:ASAP3)*, generated in this study, are available upon request to the corresponding author. Pre-processed functional 2-photon imaging data, natural imaging data, EM-data, HC circuit modelling data, associated summary statistics, and all code for the model and the statistical analysis of the experimental data are freely available on DataDryad (<https://doi.org/10.5061/dryad.pzgmsbcmk>) and further archived via the relevant links on <http://www.badenlab.org/resources> and <http://www.retinal-functomics.net>. Code for the model and the statistical analysis of the experimental data are also available on Github (https://github.com/berenslab/cone_colour_tuning). Natural imaging datasets were published previously as part of (Nevala & Baden, 2019, Zimmermann et al, 2018).

Author contributions

TY, PBa and TB designed the study, with input from CS, FKJ and PBe. TY generated novel lines and performed 2-photon data collection and pre-processing. TY also performed anatomical imaging and EM-tracing. TY and CS analysed anatomical data. PBa built the light-stimulator with input from FKJ. PBa and TB performed natural imaging data analysis, with input from PBe. CS performed computational modelling of the HC-cone circuit with input from PBe. CS analysed voltage recordings with input from PBe. CS, TY, PBa and TB performed general statistical analyses, with help from PBe. FSP provided early access to ASAP plasmids. TB wrote the manuscript with inputs from all authors.

Declaration of Interests

The authors declare no competing interests.

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Supplemental Materials

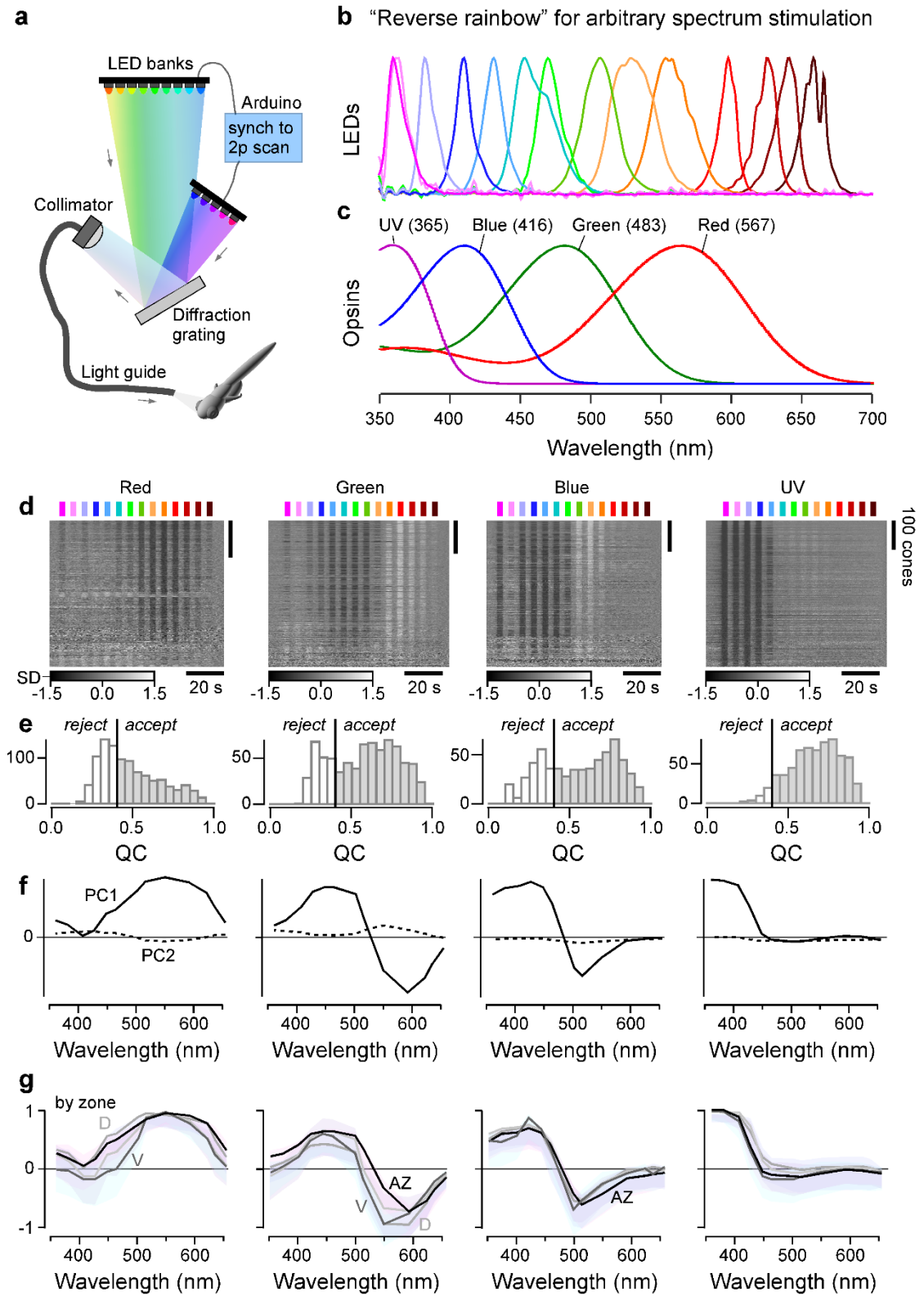


Fig S3.1: Hyperspectral stimulation under 2-photon and quality filtering of cone-recordings (related to Figure 3.1).

a, Schematic of the stimulator, inspired by (Belusic et al, 2016): Two banks holding a total of 14 spectrally distinct and collimated LEDs are pointed at a diffraction grating. A spectrally narrowed fraction of each LED's light is then further collimated into a light guide and presented as full-field to the zebrafish under the 2P microscope. To prevent spectral cross-talk, the LEDs are time-interleaved with the 2P scan (Euler et al, 2019). **b**, Peak normalised spectra of the 14 LEDs measured at the sample plane. LED Powers were presented to be equal across the full spectrum, with exception of the four short-wavelength ones which were relatively attenuated to ~50% power to ameliorate UV-saturation (Methods). Moreover, the strong spectral overlap in the two shortest wavelength LEDs was exploited as an internal UV-saturation control, by tuning the second LED to ~10% output power (Methods). **c**, Govadovskii-templates of opsin absorption spectra for the four zebrafish cones (80). **d,e** heatmaps of all cone recordings of each type as indicated ($n = 1,659$), ranked by quality criterion (b) (QC, Methods) from these recordings, with a cut-off at $QC = 0.4$, as indicated. **f**, First principal components from tunings extracted from all ROIs of a given cone type (PC1 and 2 are shown). In all cone types, PC1 explains >80% of the variance in the data. Thus, we used PC1 loadings to remove outliers (Methods). **g**, Means $\pm$ SD for all ROIs that passed QC and outlier filtering, segregated by recording region (acute zone (AZ), black; dorsal (D), dark grey; nasal (N), mid grey; ventral (V), light grey). Note that most respective tunings superimpose well, indicating that cone-tunings are approximately eye-region invariant. While significant regional variations existed in all cone-types (Discussion), these were generally very small when compared to across-cone-type differences. Accordingly, for further processing we used the averages of all zones of a given cone type. Note that heatmaps (d) are time-inverted to facilitate comparison to summary plots (f,g). Greyscale bars are in z-scores.

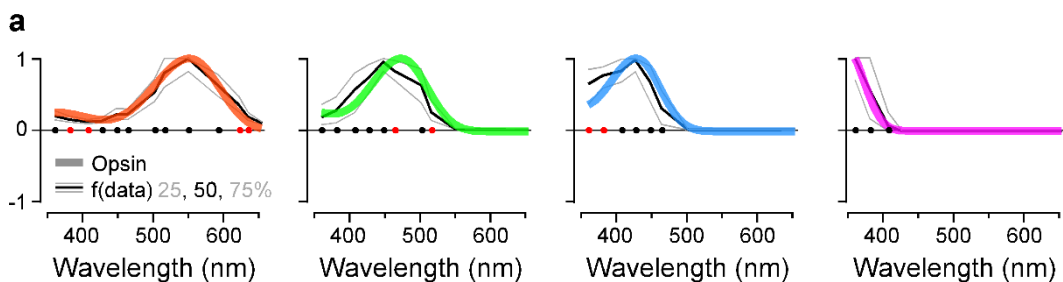


Fig S3.2: Statistical comparison of opsin tunings with in vivo cones-responses in absence of HCs (related to Figure 3.2).

a, exponential fits of the HC blockage to the opsin curves. Dots indicating for which wavelength the opsin curve lies within (black) or outside (red) 50% of the data distribution

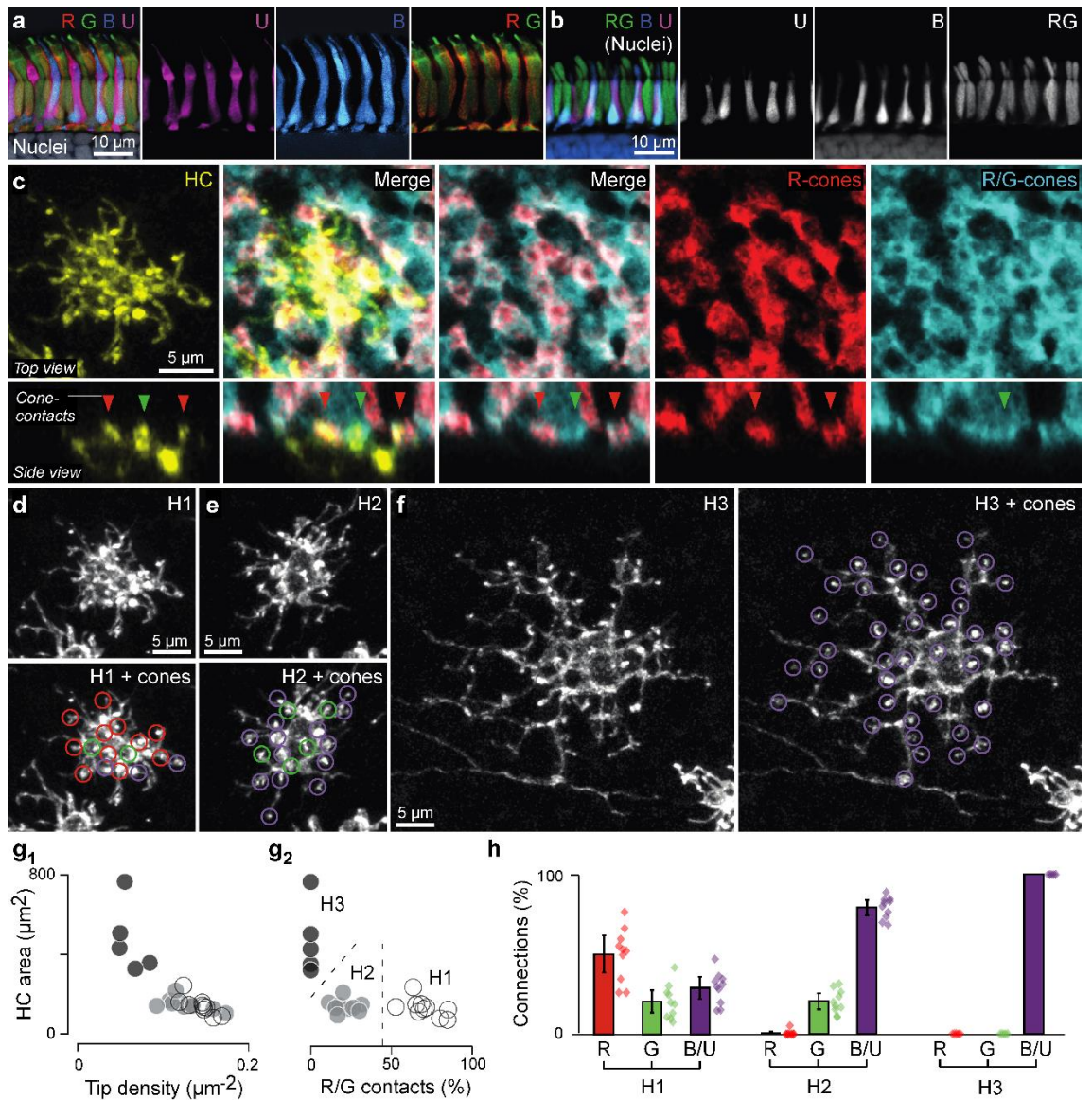


Fig S3.3: Cone-HC circuit connections by light microscopy (related to Figure 3.3).

a, vertical cross-section through outer retina, with the four cone-types individually labelled by the combination of transgenic labelling of cone types in *Tg(Opn1sw1:GFP, Opn1sw2:mCherry, thrb:Tomato)* and *zpr-1* antibody immunostaining (red, green, blue, magenta, as indicated) on a background of a DAPI nuclear stain (grey) (Methods). **b**, as (a) but now DAPI signal in specific cone types were extracted. **c**, Example single HC randomly labelled by plasmid injection into one-cell stage eggs to express membrane targeting YFP, with cone pedicles of red cones (*Tg(thrb:Tomato)*, red) and both red- and green cones labelled (*zpr-1* antibody immunostaining, cyan). This allowed directly attributing each HC contact with red-, green-cones (e.g. arrowheads) or others (blue- or UV-cone). **d-f**, example single HCs identified as H1 (d), H2 (e) and H3 (f), with cone-

type contacts indicated. **g**, HC dendritic area in relation to tip density (g_1) and percentage of red- and green-cone contacts (g_2) for $n = 25$ HCs allowed splitting HCs into 3 groups, here allocated to H1-3 as indicated. **h**, Relative cone contributions to the three HCs.

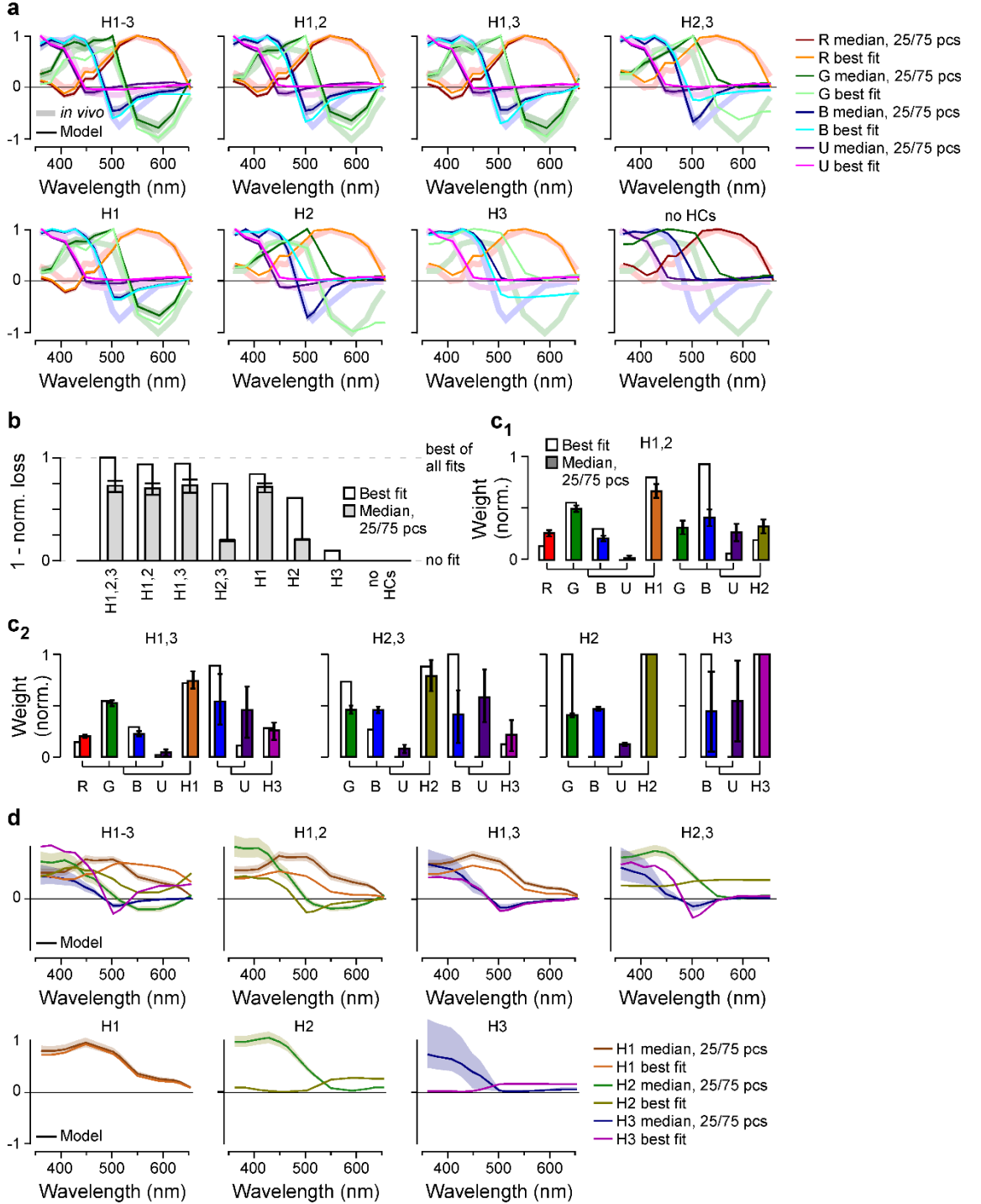


Fig S3.4: Additional HC model quantification (related to Figure 3.4).

a, cone tuning functions (as in Fig 3.2b) that emerge from models comprised of different HC combinations as indicated, with best fit, median and 25/75 percentiles

plotted on top of the measured *in vivo* cone tunings (thick shaded lines). **b-e**, normalised loss (b), distribution of weights (c) and emergent HC tunings (d) for all modelled HC-combinations (as Fig 3.4c-e, respectively).

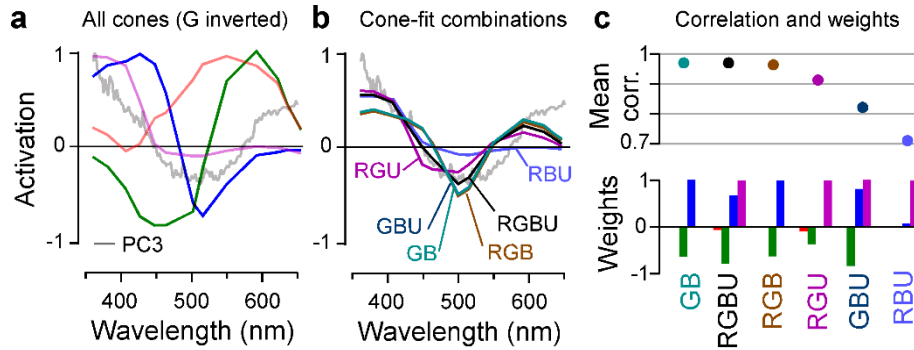


Fig S3.5: Linking zebrafish cone-tunings to PC3 (related to Figure 3.5).

a, mean *in vivo* cone-spectra superimposed on PC3 (from Fig 3.5i, n), with green-cone tuning y-inverted for illustration. **b,c**, linear combinations of cone-tunings as indicated fitted to match PC3 based on least squares (b), and comparison of their performance in doing so (c). Note that GB, RGBU and RGB combinations all yield similar quality fits ($p \sim 0.97$, top). In each case green-cones needed to be inverted relative to blue-cones (weights, bottom). Validation is based on the test data, as in Fig 3.6a,b.

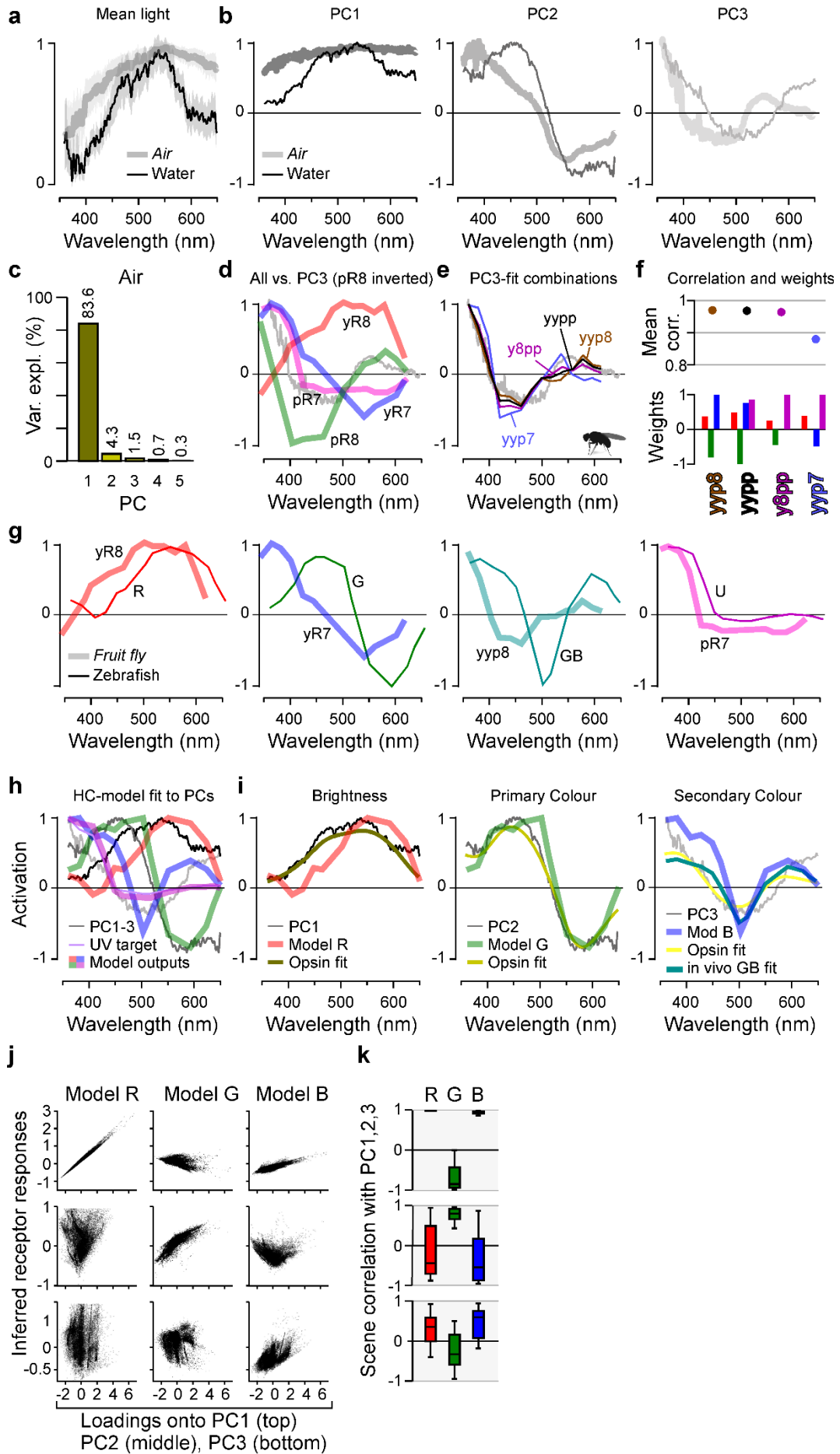


Fig S3.6: Spectral processing in Drosophila, and a linear HC network fails to produce PC-like tuning in zebrafish blue-cones (related to Figure 3.6).

a, Mean $\pm$ SD of the full spectrum of natural light in air (grey) and water (black) and **b**, the respective first three principal components that emerge. Note that “air-PCs” are systematically short-wavelength shifted compared to “water-PCs”. **c**, Variance explained by the first five PCs in the air-dataset. **d-f**, (as Fig S3.5, S3.2), here for fly R7/8 photoreceptors fitting to “air-PC3”. Note that the best fits opposed yR7+yR8 against pR8 (hence “yyp8”). Validation on the test data, as in Fig 3.6a,b. **g**, superposition of “functionally homologous” fly- and zebrafish-photoreceptor tunings that capture PC1, PC2, PC3, respectively (from left to right). The “unused” zebrafish UV-cones and fly pR7 are superimposed in the final panel. Note that these four Drosophila curves are reminiscent of relatively short-wavelength shifted versions of the zebrafish curves. **h**, Horizontal cell model fit (cf. Fig 3.4) when optimised to match red-, green- and blue-cones to PC1, PC2 and PC3, respectively. UV-cones were fitted to their own *in vivo* spectrum (as before), and **i**, respective matches superimposed. As before (cf. Fig 3.5n), log-opsin-fit curves are added for reference. **j**, Comparison of measured *in vivo* tunings (thin lines) with respective HC-model outputs (thick lines). **k**, Evaluation of the above HC-model output tunings of red-, green- and blue-cones for capturing PCs1-3 (cf. Fig 3.6a,b). Note that blue-cones still correlate with PC1 rather than PC3, while now green-cones fail to capture PC2.

	H1 weights (%)					H2 weights (%)				H3 weights (%)		
	R	G	B	U	H1	G	B	U	H2	B	U	H3
D	17.8	61.1	21.1	0	52.5	52.3	47.7	0	32.8	83.3	16.7	14.7
N	26.5	47.2	21	5.3	48.6	68.6	31.4	0	26.8	100	0	24.7
AZ	43.3	31.2	25.5	0	38.4	71.1	26.7	2.2	26.8	100	0	34.8
V	27.9	46.2	19.3	6.6	43.6	50.4	49.6	0	31.7	72.6	27.4	24.7
All	25.7	49.7	19.4	5.2	55.8	64.9	35.1	0	33.5	90.3	9.7	10.7

Supplemental Table S3.1 – related to Figure 3.7. Horizontal cell model outputs when individually computing spectral matches based on recordings taken only from one eye-region at a time. We used region specific cone recordings (Fig S3.1g) as target for the model output as well as region specific model input from the experimental HC blocked condition (Fig 3.2b). The relative weights of the best fits are shown, “all” refers to the data presented in Fig 3.4d, “best fit” as in Fig 3.4d, the posteriors (not shown) allow for

some variability of the model weights, but the overall connectivity motifs stay the same across eye-regions.

4. Bipolar Cell Terminals: diverse chromatic processing

Since this is so, it is clear that light through the infinite multiplication of itself extends matter into finite dimensions that are smaller and larger according to certain proportions that they have to one another, namely, numerical and non-numerical.

Robert Groesseteste, On Light or the Beginning of Forms

Spectral inference reveals principal cone-integration rules of the zebrafish inner retina

Philipp Bartel¹, Takeshi Yoshimatsu¹, Filip K. Janiak¹, and Tom Baden^{1,2\$}

1, School of Life Sciences, University of Sussex, Biology Road, BN1 9QG, Brighton, UK; 2, Institute of Ophthalmic Research, University of Tübingen, Elfriede-Aulhorn-Strasse 7, 72076, Tübingen, Germany.

^{\$}Correspondence to t.baden@sussex.ac.uk

Twitter: @NeuroFishh

Summary

Retinal bipolar cells integrate cone-signals at dendritic and axonal sites. The axonal route, involving amacrine cells, remains largely uncharted. However, because cone-types differ in their spectral sensitivities, insights into bipolar cells' cone-integration might be gained based on their spectral tunings. We therefore recorded *in vivo* responses of bipolar cell presynaptic terminals in larval zebrafish to widefield but spectrally resolved flashes of light and mapped the results onto spectral responses of the four cones. This "spectral-circuit-mapping" allowed explaining ~95% of the spectral and temporal variance of bipolar cell responses in a simple linear model, thereby revealing several notable integration rules of the inner retina. Bipolar cells were dominated by red-cone inputs, often alongside equal sign inputs from blue- and green-cones. In contrast, UV-cone inputs were uncorrelated with those of the remaining cones. This led to a new axis of spectral opponency where red-/green-/blue-cone "Off" circuits connect to "natively-On" UV-cone circuits in the outermost fraction of the inner plexiform layer – much as how key colour opponent circuits are established in mammals. Beyond this, and despite substantial temporal diversity that was not present in the cones, bipolar cell spectral tunings were surprisingly simple. They either approximately resembled both opponent and non-opponent spectral motifs already present in the cones or exhibited a stereotyped non-opponent broadband response. In this way, bipolar cells not only preserved the efficient spectral representations in the cones, but also diversified them to set up a total of six dominant spectral motifs which included three axes of spectral opponency.

Keywords. Retina, zebrafish, bipolar cell, cone-photoreceptor, amacrine cell, colour vision, spectral processing

Introduction

For colour vision, retinal circuits combine and contrast the signals from spectrally distinct types of photoreceptors (Baden & Osorio, 2019). For this, our own trichromatic vision uses spectral signals along two main opponent axes: “blue-yellow” and “green-red” (Jacobs, 1996, Field et al, 2010, Dacey, 2000, Buchbaum & Gottschalk, 1983). Of these, blue-yellow comparisons are based on ancestral cone-type selective retinal circuits that differentially contact SWS1- (“blue”) and LWS-cones (“green/red”, aka. “yellow”), while reliably contrasting “green-red” is thought to require the central brain (Baden & Osorio, 2019, Buchbaum & Gottschalk, 1983, Dacey & Packer, 2003, Pasupathy et al, 2020). This is because primate “green”- and “red-cones” emerged from a relatively recent LWS gene duplication that enabled new green sensitivity in some LWS-cones, however without providing a known means for postsynaptic retinal circuits to distinguish between “green” and “red” LWS-cone variants (Field et al, 2010, Jacobs & Rowe, 2004). Accordingly, in our own eyes, one axis of spectral opponency arises in the retina, and a second is probably decoded only in the brain.

In contrast, most non-mammalian vertebrate lineages, including fish, amphibians, reptiles, and birds, retain the full complement of ancestral cone-types based on four opsin-gene families: SWS1 (UV-cones), SWS2 (blue-cones), RH2 (green-cones), LWS (red-cones) (Baden & Osorio, 2019, Baden, 2019, Meier & Nelson, 2018, Musilova et al, 2021). Each of these four ancestral cones provide type-specific extracellular matrix proteins that developmental programmes use to build cone-type selective circuits in the outer retina (e.g. zebrafish (Li et al, 2012, Li et al, 2009, Klaassen et al, 2016, chicken (Yamagata et al, 2021, Gunther et al, 2021, Seifert et al, 2020)). Accordingly, in these non-mammalian lineages, the expectation is that up to tetrachromatic colour vision should be possible based on stereotyped cone-opponent ancestral circuits that are specified during development, without a necessity for building additional spectral opponencies in the brain. In agreement, physiological recordings from retinal neurons in cone-tetrachromatic species including turtles (Rocha et al, 2018) and diverse species of fish (Baden, 2021, Zimmermann et al, 2018, Daw, 1968, Guggiana et al, 2021, Zhou et al, 2020, Torvund et al, 2017) consistently revealed a rich complement of complex spectral signals, including diverse spectral opponencies.

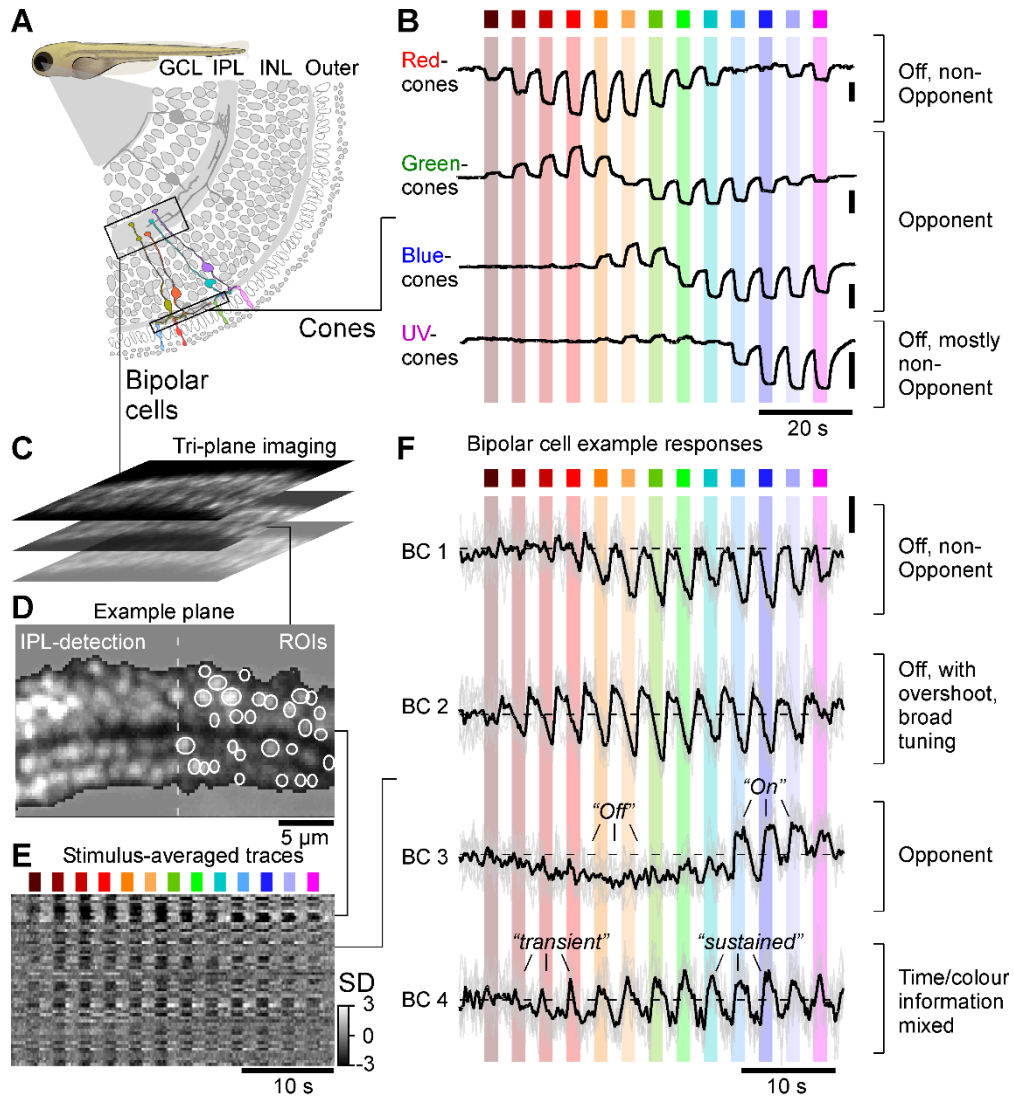


Fig 4.1: Measuring high-spectral resolution tuning curves in zebrafish bipolar cells.

A, Schematic of the larval zebrafish retina, with cone-terminals in the outer retina and bipolar cell (BC-) terminals in the inner retina highlighted. **B**, Mean calcium-responses of red- green-, blue- and UV-cone terminals to a series of 13 spectrally distinct widefield flashes of light as indicated (data from Yoshimatsu et al, 2021). Note that for clarity the response to a 14th “low-power-control” UV-LED was graphically removed compared to the original publication. **C-F**, Illustration of recording strategy for BC-terminals in the inner plexiform layer (IPL), and exemplary results. An optical tri-plane approach (**C**, top) was used to simultaneously record from three planes of larval zebrafish BC-terminals expressing SyGCaMP6f by way of two-photon imaging coupled with remote focussing (Methods). From here, we automatically placed regions of interest (ROIs) and detected the boundaries of the IPL (**D**, Methods). Time traces from all ROIs in a recording plane were z-scored and averaged across 3-5 response repeats of the full stimulus sequence (**E**). Example traces from individuals ROIs (**F**) are shown

as individual repeats (grey) and averages across repeats (black). Zebrafish larva schematic (A) by Lizzy Griffith. See also Figure S4.1.

However, what the dominant opponencies are, and how they are built at the circuit level remains incompletely understood in any cone-tetrachromat vertebrate (Baden, 2021). This is in part because already horizontal cells in the outer retina functionally interconnect and potentially retune cone-types (Meier et al, 2018, Klaassen et al, 2016, Kamermans et al, 1991, Kamar et al, 2019, Connaughton & Nilson, 2010), thus limiting the possibility of making inferences about spectral processing based on recordings from downstream neurons. To address this, we recently measured the *in-vivo* spectral tuning of the synaptic outputs from the four cone-types in larval zebrafish using spatially widefield but spectrally narrow flashes of light (Yoshimatsu et al, 2021). This revealed that red-cones are non-opponent, green- and blue-cones are strongly opponent with distinct zero crossings (~523 and ~483 nm, respectively), and UV-cones are weakly opponent with a zero crossing at ~450 nm. Accordingly, in larval zebrafish already the cone-output provides up to three axes of spectral opponency (Baden, 2021, Yoshimatsu et al, 2021). However, the opponent axis provided by UV-cones was weak, which left its role in zebrafish colour vision unclear. Moreover, in view of expected extensive mixing of cone-signals in downstream circuits (Li et al, 2012, Euler et al, 2014), if and how the cones' spectral axes are propagated downstream remains unknown.

Accordingly, we asked how downstream retinal circuits make use of the spectrally complex cone signals to either consolidate or to retune their spectral axes for transmission to the brain. For this, we used two-photon (2P) imaging to measure spatially widefield but spectrally highly resolved tuning functions at the level of retinal bipolar cell (BCs) presynaptic terminals in the inner retina. This strategy was previously used to establish the spectral tunings of the cones (Yoshimatsu et al, 2021, Bartel et al, 2021), thus facilitating direct comparison.

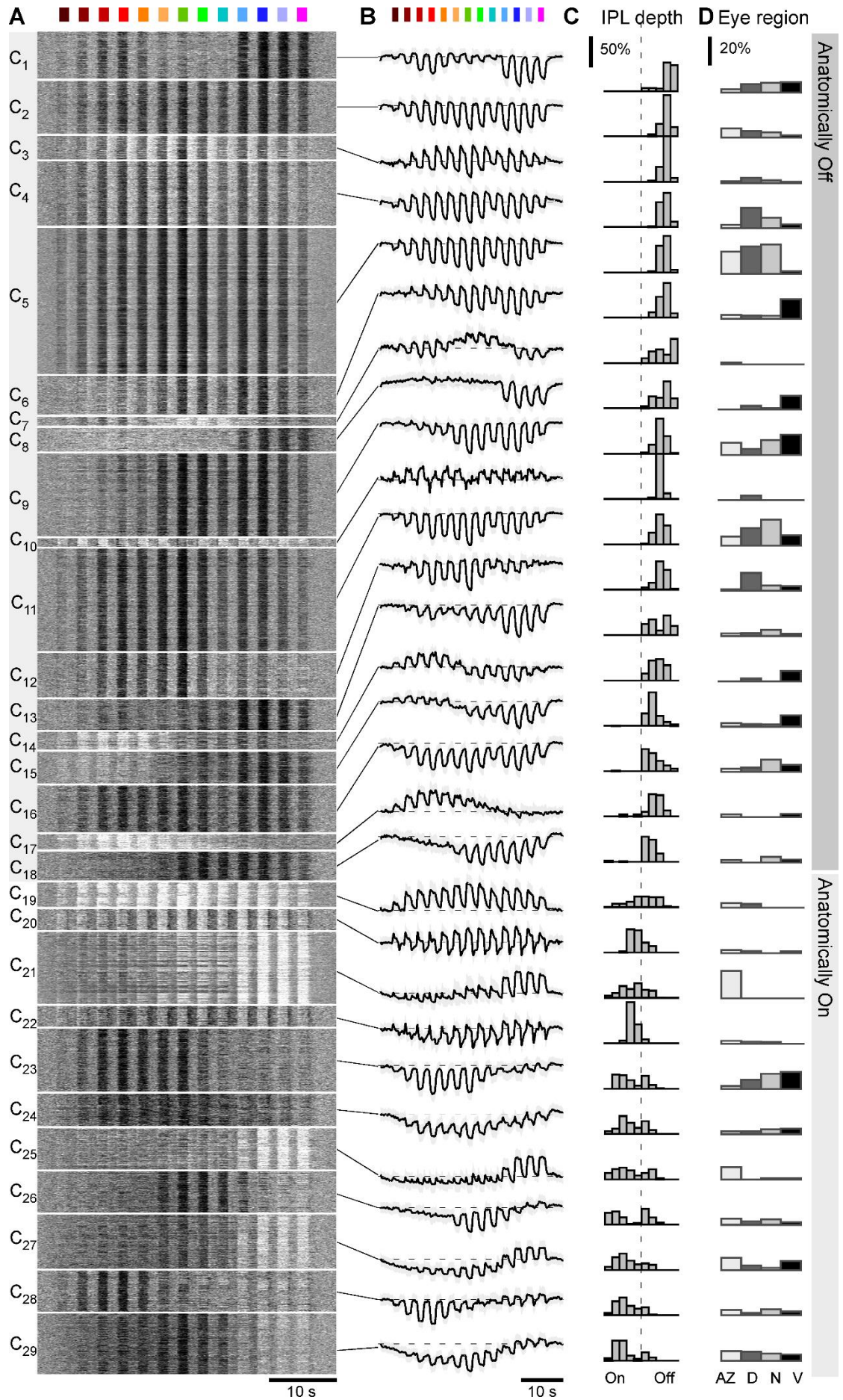


Fig 4.2: Clustering into 29 functional BC-types.

A-D, Overview of the result from unsupervised clustering of all BC-data recorded as shown in Figure 1 that passed a minimum quality index ($QI > 0.4$, Methods). For each cluster, shown are the individual BC-mean responses as heatmaps (A), the corresponding cluster means and SD shadings, with approximate baseline indicated in dashed (B), distribution of ROI positions in the IPL (C) and each cluster's distribution across the four recording regions within the eye (D, from left: acute zone, dorsal, nasal, ventral). Histograms in (C) are area-normalised by cluster, and in (D) by recording region. Clusters are ordered by their average anatomical position in the IPL, starting from the border with the inner nuclear layer (cf. C). The coloured symbols indicate the overall spectral group as assigned later (cf. Fig 4.5F-K).

We find that all three spectral axes already set-up by the cones are conserved at the level of BC presynaptic terminals, and no new axes are created. However, the “UV-red” axis was notably boosted and diversified into numerous variants of either polarity via new opponent circuits that derive from red-/green-/blue-Off-circuits connecting to UV-On-circuits. The remaining non-opponent BCs were either broadly tuned, likely built by pooling signals from all four cone types, or essentially resembled the tunings of red- and/or UV-cones in isolation. Beyond spectral tuning, bipolar cells showed a rich complement of temporal features that were absent in cones, which were notably intermixed with spectral information.

Taken together, larval zebrafish BC-circuits for colour vision therefore directly built upon the existing cone-tunings rather than set up fundamentally new opponencies, while at the same time adding substantial temporal complexity to the retinal code.

Results

A complex interplay of spectral and temporal signals amongst BCs. To establish *in vivo* spectral tuning functions at the level of individual presynaptic terminals of bipolar cells (BCs) in the inner retina, we imaged light-evoked calcium responses from 6-7 days post fertilisation (*dpf*) RibeyeA:SyjGCaMP7b zebrafish under two-photon (2P) using established protocols (Zimmermann et al, 2018, Rosa et al, 2016, Dreosti et al, 2009) (Methods). To record from 100s of individual BC terminals in parallel, we used a non-telecentric triplane imaging approach (Janiak et al, 2019) (Methods). For light-stimulation, we used the same system and protocol previously employed to determine cone-tunings (Yoshimatsu et al, 2021) (Fig 4.1A,B). In brief, light from 13 spectrally distinct LEDs was collected by a collimator after reflecting off a diffraction grating which served to narrow individual LED spectra reaching the eye (Belusic et al, 2016). From here, stimuli were presented to the fish as widefield but spectrally narrow flashes of light (1.5 s On, 1.5 s Off, starting from “red” and sweeping towards UV; Methods). One example recording from BC terminals is illustrated in Fig 4.1C-E alongside averaged cone-responses to the same stimulus (Fig 4.1B) taken from Yoshimatsu et al, 2021. In short, each recording plane was automatically processed to detect the boundaries of the inner plexiform layer (IPL, Fig 4.1D, left) and to place regions of interest (ROIs) based on pixel-wise response coherence over consecutive repeats (Fig 4.1D, right, Methods). From here, fluorescence traces from each ROI were extracted, detrended, z-scored, and averaged over typically 7-8 stimulus repetitions (Fig 4.1D,E). This revealed a great diversity in both the spectral and the temporal composition of responses amongst BCs. For example, some ROIs were entirely non-opponent but differed in their spectral tuning and in the degree to which they “overshot” the baseline between stimulus presentations (Fig 4.1F, compare ROIs labelled BC1 and BC2). Other ROIs such as the one labelled BC3 were spectrally opponent, here exhibiting Off-signals to mid-wavelength stimulation but On-signals to UV-stimulation. Finally, some ROIs including the one labelled BC4 exhibited different temporal responses to long- and short-wavelength stimulation.

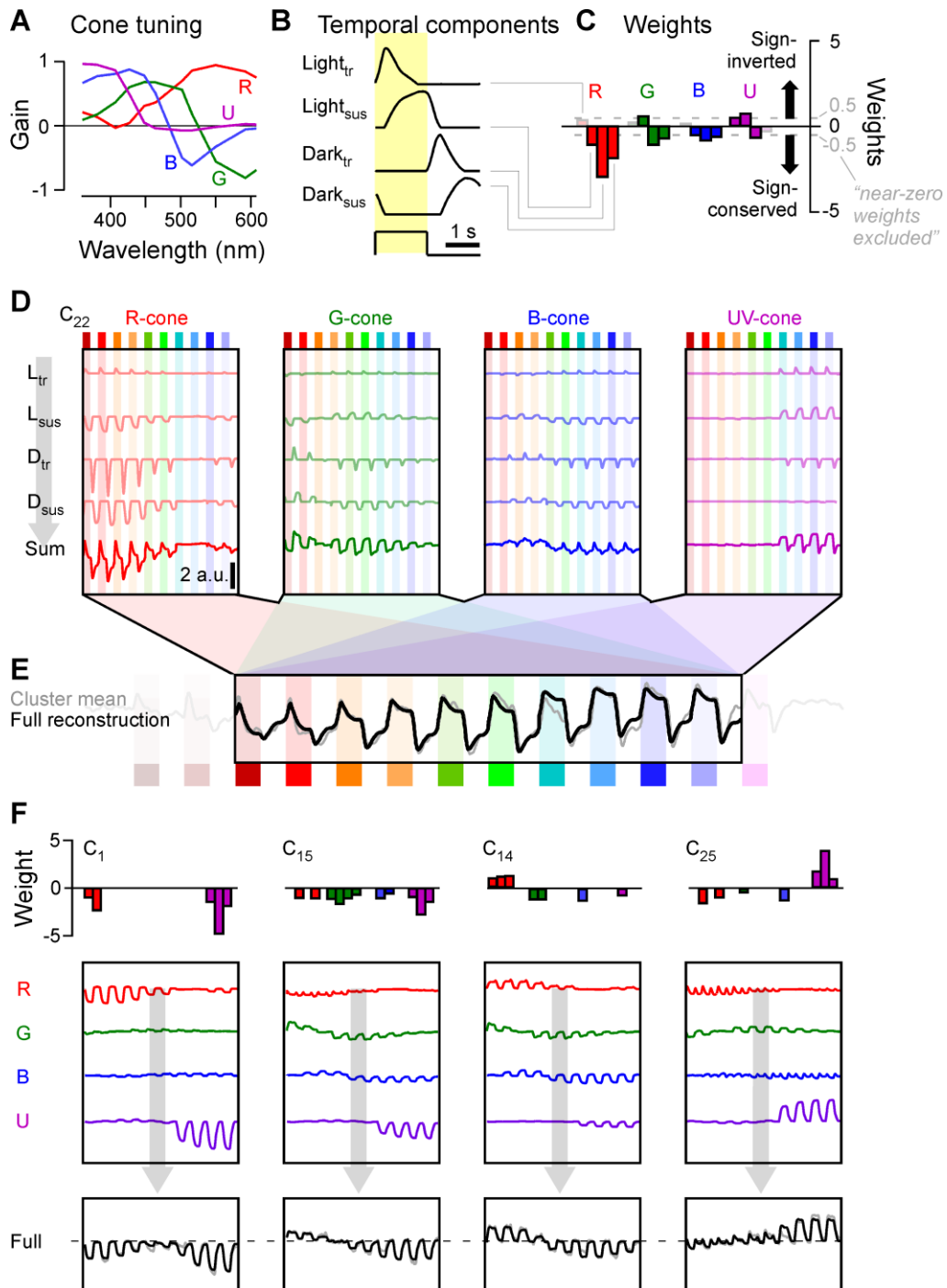


Fig 4.3: Reconstructing bipolar cell responses from cones.

A-E, Summary of the reconstruction strategy for example cluster C₂₂ (for details see Methods). Each BC-cluster reconstruction is based on the linear combination of the spectral tunings functions of the four cone-types (A, from Yoshimatsu et al, 2021) with four stereotyped temporal components associated with individual light flashes (B), yielding $4 \times 4 = 16$ weights (C). Weights are shown in blocks of temporal component weights (from left: Light-transient, Light-sustained, Dark-transient, Dark-sustained) associated with each cone (indicated by the corresponding colours). Bars above zero

indicate sign-inverted (“On-”) weights, while bars below zero indicate sign-conserved (“Off-”) weights. The corresponding full expansion of this reconstruction is shown in (D). Individual combination of each cone’s tuning function (A) with each temporal component (B), scaled by their corresponding weight (C), yields sixteen “sub-traces” (D, upper four traces in each of the four panels, labelled L_{tr} , L_{sus} , D_{tr} , D_{sus}). Summation of each cone’s four sub-traces yields that cone’s total contribution to the cluster (D, bottom traces, labelled “sum”). Finally, summation of the four cone-totals yields the full reconstruction (E, black trace), shown superimposed on the target cluster mean (grey). **F**, as A-E, but showing only the weights (top) cone-totals (middle) and full reconstructions (bottom) for another four example clusters (from left: C_1 , C_{15} , C_{14} , C_{25}). Further detail on reconstructions is shown in Fig S4.2, and all cluster’s individual results are detailed in Data S4.1.

Because stimuli were always presented in spectral sequence, which might cause systematic adaptation, we also performed a small number of control experiments with a reduced stimulus set, where we directly compared responses of the same ROIs to ordered and to pseudorandomised stimulus sequences (Fig S4.1A-G). This showed that both approaches gave very similar responses, suggesting that spectral adaptation was not a major feature in our recordings. We recorded responses from a total of $n = 72$ triplane scans in $n = 7$ fish, across four major regions of the eye: Acute Zone (AZ), Dorsal (D), Nasal (N), and Ventral (V). From here, $n = 6,125$ ROIs ($n_{AZ,D,N,V} = 2,535, 1,172, 1,889, 529$, respectively) that passed a minimum response quality criterion (Methods) were kept for further analysis. Next, we clustered BC responses using a mixture of Gaussian model as described previously (Zimmermann et al, 2018, Zhou et al, 2020, Baden et al, 2016, Franke et al, 2017) (Methods). This yielded 29 functional BC-clusters (Fig 4.2A,B), here arranged by their mean stratification position in the IPL (Fig 4.2C). If and how this relatively large number of functional BC-clusters maps onto veritable BC ‘types’ (Euler et al, 2014) remains unknown. For comparison, previous studies described 25 functional (Zimmermann et al, 2018) and 21 anatomical (Li et al, 2012) BCs, however a deeper census of zebrafish BC-types, for example based on additional data from connectomics (Behrens et al, 2016) and/or transcriptomics (Shekhar et al, 2016) remains outstanding.

Consistent with previous work that was based on a different stimulus with lower spectral resolution (Zimmermann et al, 2018), zebrafish BC-clusters were highly diverse, and many exhibited a regional bias to one or multiple parts of the eye (Fig 4.2D). For example, several UV-dominated clusters showed a clear regional bias to the acute zone (e.g. $C_{21,25}$) and/or the ventral retina (e.g. $C_{6,27}$), while many broadband

clusters were distributed approximately homogeneously across the eye except ventrally (e.g. $C_{2,5}$). However, with our current focus on BC-spectral tunings, we did not further analyse this eye-wide regionalisation.

Overall, BC-clusters differed strongly in their wavelength selectivity. For example, clusters C_1 and C_2 both hyperpolarised in response to all tested wavelengths, but C_2 was tuned broadly while C_1 exhibited a notable dip in response amplitudes at intermediate wavelengths. Other clusters exhibited clear spectral opponency. For example, clusters C_{26-29} all switched from Off-responses to long wavelength stimulation to On-responses at shorter wavelengths. A single cluster (C_7) exhibited a spectrally triphasic response. BCs also differed in their temporal responses. For example, while cluster C_2 consistently responded in a sustained manner, cluster C_3 responses were more transient and overshoot the baseline between light-flashes. Finally, diverse spectral and temporal response differences did not only exist between BC clusters, but also within. For example, cluster C_6 switched from transient responses during long-wavelength stimulation to sustained responses during short-wavelength stimulation. In some cases, such intermixing of spectral and temporal encoding in a single functional BC-cluster could be quite complex. For example, cluster C_{21} switched from small transient On-Off responses via intermediate amplitude transient-sustained On-responses to large amplitude sustained-only On-responses in a wavelength-dependent manner.

Overall, in line with connectivity (Li et al, 2012, Connaughton et al, 2004) and previous functional work, both the spectral (Zimmermann et al, 2018, Zhou et al, 2020, Torvund et al, 2017) and the temporal diversity (Zimmermann et al, 2018, Zhou et al, 2020, Torvund et al, 2017, Rosa et al, 2016, Connaughton & Nelson, 2000, Connaughton & Maguire, 1998) of larval zebrafish BCs long exceeded that of the cones, which at the level of presynaptic calcium were generally sustained (Yoshimatsu et al, 2021), and which only exist in four spectral variants (cf. Fig 4.1B).

Linear cone-combinations using four temporal components can account for BC responses. We next explored if and how these BC cluster-means (Fig 4.2B) could be explained based on cone responses (Yoshimatsu et al, 2021) (Fig 4.3, cf. Fig 4.1B). For this, we implemented a simple linear model (Methods) based on the following considerations.

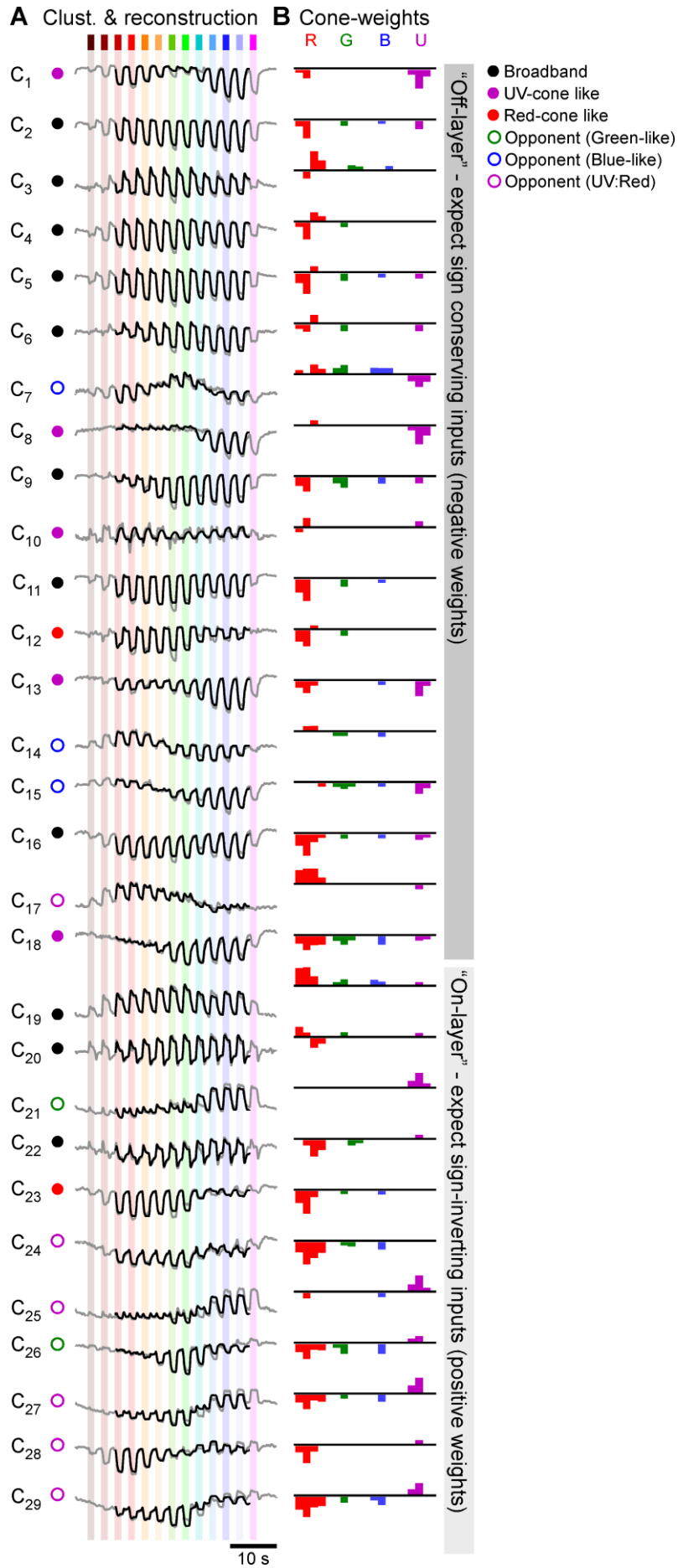


Fig 4.4: A functional overview of cone-bipolar cell mappings.

A,B, Overview of all BC-cluster means (A, grey traces, cf. Fig 4.2B) and their full reconstructions based on the strategy detailed in Fig 4.3 (black traces). Associated weights are shown in (B). For clarity, “near-zero” weights ($\text{abs}(w) < 0.5$) are omitted. Full weights are shown in Data S4.1. Note that based on outer retinal inputs only, weights are generally expected to be sign-conserving for clusters in the traditional “Off” layer (C_1 - C_{18}), and sign-inverting in the anatomical “On” layer (C_{19} - C_{29}), as indicated on the right. The round symbols plotted next to each cluster (A) denote their allocated spectral group, as detailed in Fig 4.5F-K and associated text.

BCs may receive cone inputs by two main, non-mutually exclusive routes: directly, via dendritic contacts onto cone-pedicles in the outer retina, and indirectly, via lateral inputs from amacrine cells in the inner retina (Euler et al, 2014). A third route, via horizontal cells, has been proposed in the case of mice (Behrens et al, 2019). If such a route exists in zebrafish remains unknown.

In the outer retina, direct cone inputs are based on BC-type specific expression of glutamate receptor and/or transporter variants that are thought to be either all-sign-conserving or all-sign-inverting, but apparently never a mixture of both (Euler et al, 2014, Westheimer, 2007). Accordingly, dendritic inputs alone should only be able to produce spectral tuning functions in BCs that can be explained by same-sign cone inputs. Any BC that cannot be explained in this manner is then expected to require spectrally distinct inputs from amacrine cells. On the other hand, variations to the temporal structure of a given cones’ contribution to a BC’s response could be implemented via either route (Euler et al, 2014, Franke et al, 2017, DeVries et al, 2006) – that is, via a combination of dendritic and/or axonal inputs. Accordingly, we reasoned that for a linear transformation, each cone-type may feed into a functional BC-type via a unique temporal profile that represents the sum of all routes from a given cone to a given BC. In this way, our model effectively sought to explain each BC-cluster as a weighted sum of four spectral cone-tunings, but each of these four cone-inputs could have a unique temporal structure.

To capture the above considerations in a linear model, we combined the four-cone spectral tuning functions (Fig 4.3A, cf. Fig 4.1B) with four dominant temporal components extracted from BC responses: light-transient, light-sustained, dark-transient, and dark-sustained (Fig 4.3B, Methods). We restricted the model to capture the central ten light-stimuli (i.e. omitting the first two red-flashes and the last UV-flash) where BC-clusters generally exhibited the greatest response diversity (Fig 4.2).

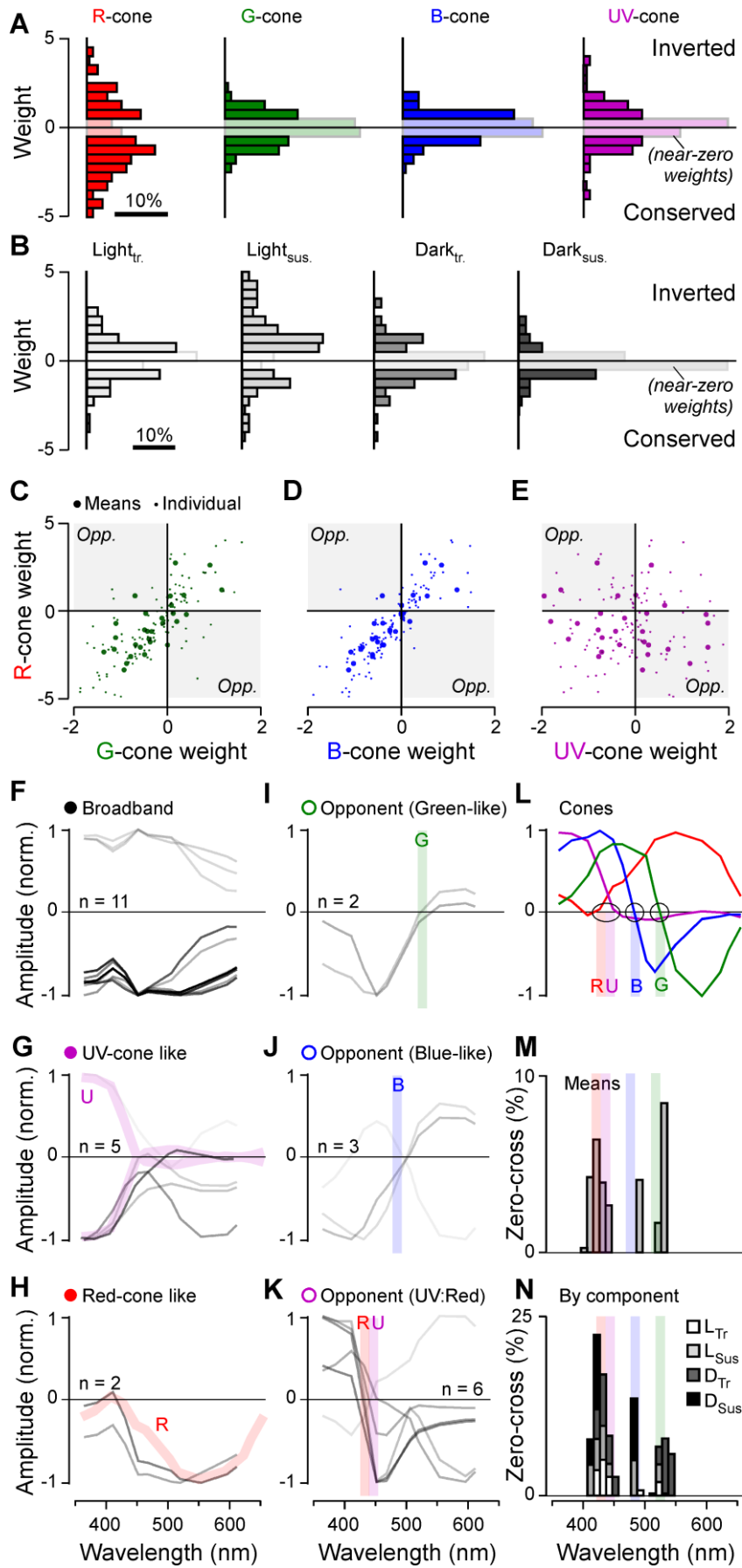


Fig 4.5: Major trends in cone-weights and spectral tunings.

A,B, Histograms of all weights associated with inputs to each of the four cones across all clusters, independent of temporal-component types (A), and correspondingly histograms of all weights associated with temporal components, independent of cone-type (B). “Near-zero” weights ($\text{abs}(w) < 0.5$) are graphically de-emphasised for clarity. All weights contributed equally to these histograms, independent of the size of their corresponding cluster. **C-E**, Scatterplots of all clusters’ weights associated with each cone plotted against each other as indicated. Large symbols denote the mean weight associated with each cone and cluster across all four temporal components (i.e. one symbol per cluster), while small symbols denote each weight individually (i.e. four symbols per cluster, corresponding to L_{tr} , L_{sus} , D_{tr} , D_{sus}). The remaining three possible cone-correspondences (G:B, G:U, B:U) are shown in Figure S3A-C. **F-K**, Peak-normalised ‘bulk’ spectral tuning functions of all 29 clusters, grouped into six categories as indicated. The strength of each line indicates the numerical abundance of ROIs belonging to each cluster (darker shading = larger number of ROIs; exact number of ROIs contributing to each cluster are listed in Data S4.1). As appropriate, spectral tuning functions of cones (cf. L) are shaded into the background, as appropriate (G,H, thick coloured traces) to illustrate the close spectral correspondences of associated cones and BCs. Similarly, for three spectrally opponent groups (I-K), the approximate positions of the corresponding cone’s zero crossings are indicated with a vertical shaded line (cf. L). **L**, Cones’ spectral tuning functions, with approximate zero-crossings (blue-/green-cones) and zero-positions (red-/UV-cones) graphically indicated. **M,N**, Histograms of zero-crossings across all BC-clusters, incorporating the abundance of ROIs belonging to each cluster. Shown are crossings of ‘bulk’ spectral tunings functions (M, cf. F-H), and of spectral tuning functions that were computed for each temporal component individually, as indicated (see also Fig S4.3F-I, and Data S4.1). Note the three prominent peaks of zero-crossing positions, approximately aligned with the zero-positions/crossings of the cones. These peaks largely disappeared when time-components were fully randomised (Fig S4.3D) or randomly permuted across cones (Fig S4.3E).

Notably in the following paragraphs, we avoid the use of the common shorthand “On” or “Off” because in view of spectral opponency already present in cones²⁷ a sign-conserving input to a BC is not categorically “Off”, and vice versa a sign-inverting input is not categorically “On”. Instead, we use the terms “light” and “dark” response, in reference to a response that occurs in the presence or absence of a light-stimulus, respectively. Also note that all extracted spectral tuning functions (e.g. Fig 4.3A) are x-inverted compared to the time-axes in recordings and reconstructions (e.g. Fig 4.3D,E).

This was done because recordings were performed from long- to short wavelength stimuli, but spectral tuning functions are conventionally plotted from short- to long-wavelengths. Weights were scaled such that the mean of their magnitude equalled one, with weights <0.5 (“near-zero”) excluded from the summary plots for visual clarity. Full weights, including a detailed overview of each cluster, are available in Data S4.1.

Figures 4.3C-E illustrate the intermediate steps (Fig 4.3C,D) and final output (Fig 4.3E) of the model for example cluster C_{22} . This functional BC-type was broadly tuned but switched from transient responses to long wavelength stimulation to more sustained responses at shorter wavelengths (Fig 4.3E, grey trace, cf. Fig 4.2A,B). To capture this behaviour (Fig 4.3E, black trace), the model drew on all four cones (Fig 4.3C), however with a particularly strong sign-conserved contribution from red-cones (Fig 4.3C, left). Here, the model placed a strong sign-conserving weight onto the dark-transient (D_{tr}) component of the red-cone (Fig 4.3D, left, third trace). The strength and sign of this weight is illustrated in Fig 4.3C (third downwards facing red bar). In addition, the model also placed weaker sign-conserving weights onto the dark-sustained (Fig 4.3D, left, fourth trace) and light-sustained (second trace) components, and a weak sign-inverted weight onto the dark-transient component (first trace). Summation of these four kinetic components yielded the total modelled red-cone contribution to this cluster (Fig 4.3D, bottom trace).

The same principle was applied across the remaining three cones, yielding a total of sixteen (four cones times four temporal components) weights per cluster (cf. Fig 4.3C). In the example presented, weights were mostly sign-conserving (facing downwards). However, to capture the relatively complex temporal dynamics of this cluster, which systematically overshoot the baseline between flashes, the model also drew on a number of weaker sign-inverted weights (facing upwards), for example for all light-transient components.

Fig 4.3F illustrates mean outputs of the model for another four example clusters with diverse spectral and temporal behaviours. Of these, the spectrally bimodal but “temporally simple” response profile of C_1 was well-approximated by all sign-conserving inputs from red- and UV-cones (Fig 4.3F, left). Similarly, the spectrally opponent behaviour of C_{15} could be captured by all-sign-conserving inputs from all four cones (Fig 4.3E, second panel). Accordingly, as expected from the cone-tunings, generating opponent responses at the level of BC terminals does not categorically require new sign-opposition in the inner retina – instead, the opponency can simply be inherited from the cones. Nevertheless, not all opponent BC responses could be explained in

this manner. For example, opponent cluster C_{14} required sign-inverted inputs from red-cones but sign-conserving inputs from green-, blue- and UV-cones (Fig 4.3E, third panel). Finally, even the more complex spectral and temporal BC-clusters could be well-approximated by relatively simple cone-mixtures. For example, C_{25} was captured by combining sign-conserved light- and dark-transient inputs from red- and blue-cones with mostly sustained and sign-inverted inputs from UV-cones (Fig 4.3E, rightmost).

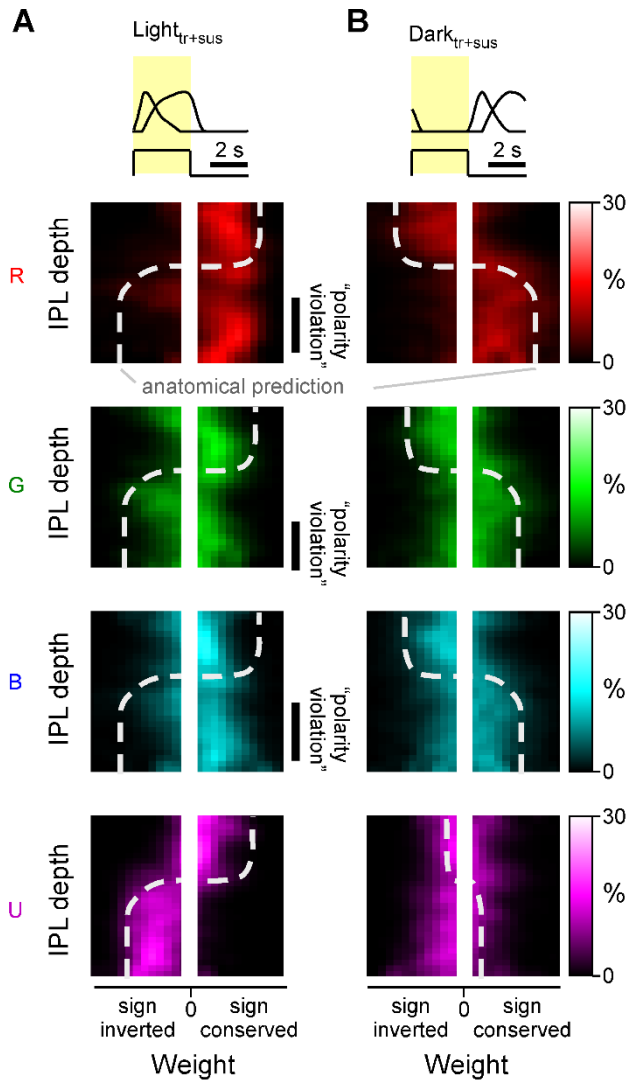


Fig 4.6: Cone-weight distribution across the inner plexiform layer.

A,B, Two-dimensional histograms of weights (x-axes) associated with each cone resolved by IPL position (y-axes). Brighter colours denote increased abundance. For simplicity, the weights associated with the light (L_{tr} , L_{sus}) and dark-components (D_{tr} , D_{sus}), are combined in panels A and B, respectively. Moreover, near-zero weights are not shown (central white bar in all panels). The thick white dotted lines indicate approximate expected distribution of weights based on traditional “On-Off” lamination of

the inner retina. By each panel's side, instances where this expectation is violated are highlighted as "polarity violation".

Overall, this linear fitting procedure captured ~95% of the total variance across the 29 cluster means (Fig S4.2A, Methods). Similarly, the fits also captured ~95% of the temporal detail, based on comparison of the mean power spectra of the cluster means and that of the residuals (Fig S4.2B, Methods). The full result of this process is summarised in Fig 4.4, each time showing the cluster mean (grey) and reconstruction (black) alongside weight-summaries per cone following the schema illustrated in Fig 4.3B,C. Further detail is shown in Data S4.1.

Based on the traditional separation of the inner retina into "Off-" and "On-layers"²⁸, we may correspondingly expect mainly sign-conserving (negative) weights in "Off-stratifying" clusters C_1 - C_{18} , and mainly sign-inverting (positive) weights for "On-stratifying" clusters C_{19} - C_{29} . However, this expectation was not met in several cases, for example for most of the On-stratifying clusters which nevertheless showed a general abundance of negative ("Off") weights for red-, green- and blue-cone inputs. From here, we next explored the general rules that govern overall cone-signal integration by BCs.

The inner retina is dominated by red-cone inputs. First, we computed histograms of all weights per cone (Fig 4.5A) and per temporal component (Fig 4.5B) to determine the dominant input-motifs across the population of all BCs. This revealed that overall, the amplitudes of red-cone weights tended to be larger than those of all other cones (red absolute weights $W_R = 1.82 \pm 1.22$; $W_{G,B,U} = 0.68 \pm 0.47, 0.62 \pm 0.45, 0.87 \pm 0.88$, respectively, range in SD; $p < 0.001$ for all red-combinations, Wilcoxon Rank Sum Test). This red-dominance was stable also when the four eye-regions were analysed separately ($p < 0.001$ in each case). Similarly, light-response component weights tended to be larger than dark-response component weights ($W_{LT, LS, DT, DS} = 0.94 \pm 0.75, 1.73 \pm 1.20, 0.85 \pm 0.8, 0.48 \pm 0.54$, respectively Fig 4.5B). Here, the light-sustained response components that already dominate the cones (cf. Fig 4.1B) remained largest overall also in BCs ($p < 0.001$ for all $Light_{sus}$ -combinations, Wilcoxon Rank Sum Test).

Red-, green- and blue-cone weights co-vary independent of UV-cone weights. Next, we explored the weight relationships between the four cone types across clusters. In general, a strong correlation between weights attributed to any two cone types would suggest that inputs from these cones tend to be pooled, for example by the dendrites of individual BCs contacting both cone-types. In contrast, a low

correlation or even anticorrelation between cone-weights could indicate the presence of cone-opponency.

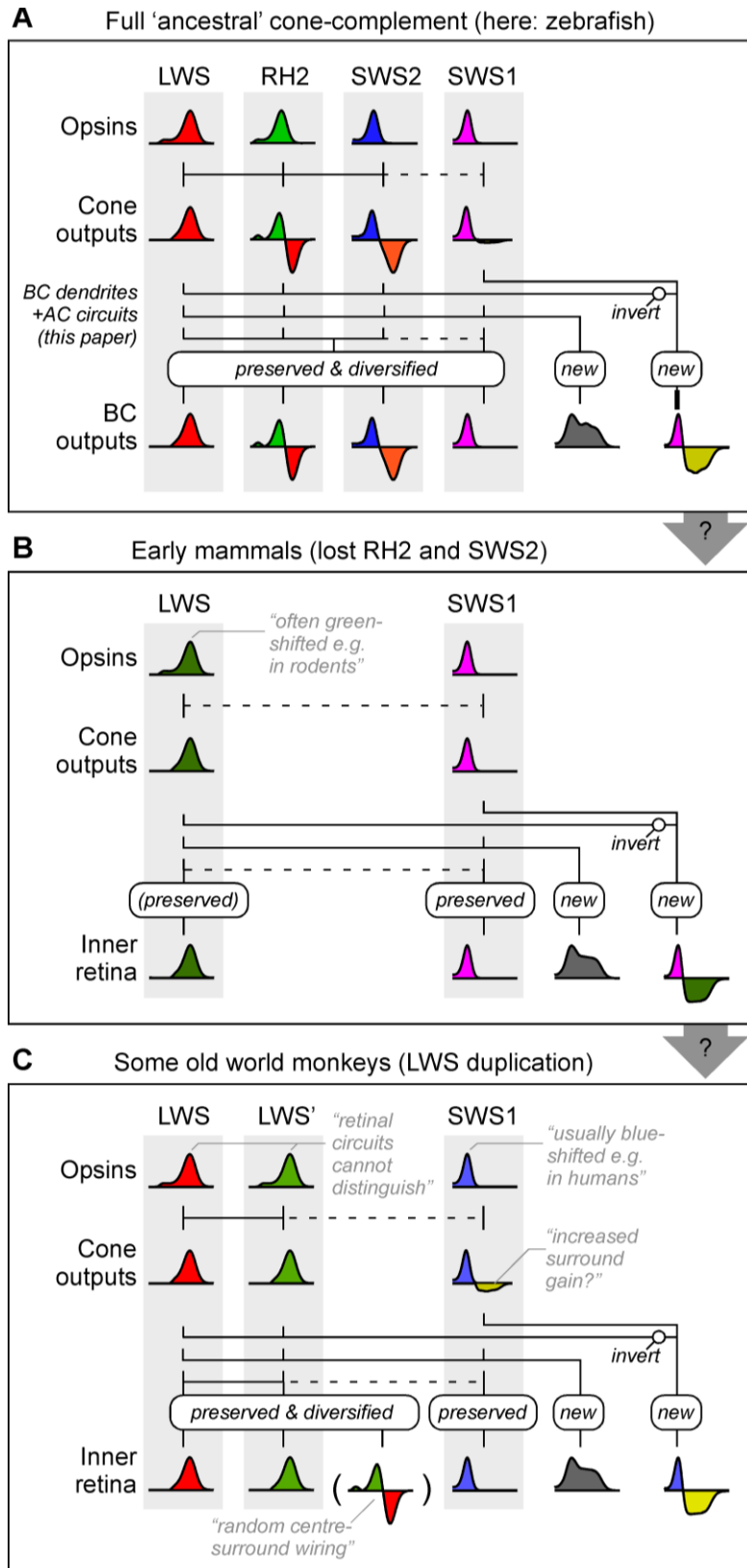


Fig 4.7: Possible links across vertebrate retinal colour circuits.

A-C, *Conceptual summary schematics of retinal circuits for colour vision in zebrafish (A), dichromatic mammals such as many rodents (B) and some trichromatic old-world monkeys such as humans (C). The coloured ‘graphs’ indicate approximate spectral tuning functions of retinal neurons in a given layer, as indicated.*

Across clusters, we found that red-cone weights strongly correlated the weights of both green- ($p = 0.73$; 95% confidence intervals (CI) 0.49/0.86, Fig 4.5C) and blue-cones ($p = 0.87$, CI 0.74/0.94, Fig 4.5D; green vs. blue: $p = 0.89$; CI 0.77/0.95, cf. Fig S4.3A). The tight association between red-, green- and blue-cone weights extended across both the all-sign inverting (bottom left) and the all-sign-conserving (top right) quadrants and comprised few exceptions in the two remaining quadrants that would indicate cone-opponency. Accordingly, zebrafish BCs did not tend to differentially combine inputs from red-, green- or blue-cones of either polarity to set up potentially new opponent-axes.

In contrast, red-cone weights were uncorrelated with UV-cone weights ($p = -0.21$, CI -0.55/0.14, Fig 4.5E, green vs. UV: $p = -0.04$, CI -0.40/0.34; blue vs. UV: $p = -0.34$, CI -0.63/0.03, see Fig S4.3B,C), with many clusters scattering across the two sign-opponent quadrants (i.e. top left, bottom right). Accordingly, reconstructing a substantial fraction of BC clusters required opposite sign inputs from red-/green/blue-versus UV-cones, suggestive of a newly set-up form of spectral opponency in the inner retina. Interestingly, in some cases, a cluster could exhibit small “Off” responses in the UV range despite using sign-inverting weights for UV-cones (e.g. C₂₃ – best seen in Data S4.1). This was possible because all cones respond to UV-light to some extent (Fig 4.1B). In some cases, the sum of inferred red-, green, and blue-cone inputs could then outweigh inferred UV-cone inputs. We next explored the spectral tuning of BC-clusters in further detail.

BC spectral responses fall into three opponent, and three non-opponent groups.

The complex interplay of temporal and spectral structure in BC-responses (Fig 4.2) meant that their spectral tuning functions could not easily be extracted directly from the BC-cluster means, for example by means of taking the area under the curve in response to each flash of light. Instead, we estimated their tuning functions based on their fitted cone-weights (cf. Fig 4.4). To this end, for each cluster we summed sixteen cone-tuning functions (based on Fig 4.3A), each scaled by the cluster’s associated sixteen weights (i.e. red-L_{tr} + red-L_{SUS} + red-D_{tr} and so on). This summarised each cluster’s ‘bulk’ response in a single spectral tuning function that gave equal weight to each of the four temporal components (Fig 4.5F-K). By this measure, 18 of the 29 BC-

clusters were non-opponent (62%, Fig 4.5F-H) and 11 were opponent (38%, Fig 4.5I-K). Here, opponency was defined as any tuning function that crossed and overshot zero at least once with an amplitude of at least 10% compared to that of the opposite (dominant) polarity peak response.

Non-opponent clusters ('closed' symbols, cf. Fig 4.4A) approximately adhered to three major groups: spectrally broad (three On- and eight Off-clusters, Fig 4.5F), approximately UV-cone-like (one On- and four Off-clusters, Fig 4.5G), and approximately red-cone-like (two Off-clusters, Fig 4.5H). Similarly, opponent clusters ('open' symbols) fell into three major groups based on the spectral positions of their zero crossings: Two green-cone-like clusters (both short_{Off}/long_{On}, crossing at 520 and 536 nm, Fig 4.5I), three blue-cone-like clusters (two short_{Off}/long_{On} crossing at 497 and 499 nm, plus the single triphasic C₇ with a dominant short_{On}/long_{Off} zero crossing at 490 nm, Fig 4.5J), and six UV-cone versus red-/green-/blue-cone opponent clusters (henceforth: UV:R/G/B, five short_{On}/long_{Off}, crossing at 416, 425, 428, 435, 448 nm, one short_{Off}/long_{On} crossing at 438 nm, Fig 4.5K). In comparison, green- and blue-cone zero-crossings, respectively (Fig 4.5L, from Yoshimatsu et al, 2021) occurred at ~523 and ~483 nm, while red- and UV-cones, respectively, approached zero between ~425 and 450 nm (Fig 4.5I-N, shadings).

The tight correspondence between opponent BC-clusters (Fig 4.5I-K) and cone-tunings (Fig 4.5L) was further illustrated by the histogram of BC-zero-crossings that also incorporated relative abundances of ROIs contributing to each cluster (Fig 4.5M). The histogram showed three clear peaks that were well-aligned to the three spectral axes set-up in the cones (shadings). Further, the histogram also retained its overall shape when the four temporal components underpinning each cluster were considered individually (Fig 4.5N). As a control, this trimodal structure disappeared when component-weights were iteratively randomised (Fig S4.3D), or when temporal-components were randomly shuffled between cones (Fig S4.3E), suggesting that the measured BC tunings emerged from non-random effective cone-inputs. In support, and despite appreciable diversity, the spectral tuning functions of the four temporal components that contributed to a given cluster tended to be positively correlated among both opponent and non-opponent clusters (Fig S4.3F-I).

Remarkably therefore, it appears that by and large, BCs tended to retain many of the dominant spectral properties of the cones rather than build fundamentally new spectral axes – all despite integrating across multiple cone types and presumably diverse inputs from spectrally complex ACs (Torvund et al, 2017). The only two notable deviations

from this observation were a highly stereotypical spectral broadening in 11 clusters (Fig 4.5F), which may be linked to outer retinal cone-pooling (Li et al, 2012), and, strikingly, the emergence of six strongly UV:R/G/B opponent clusters (Fig 4.5K).

UV-cone, but not red-/green-/blue-cone weights follow traditional IPL On-Off lamination. Finally, we asked where the inferred new form of UV:R/G/B opponency might be set-up in the inner retina (Fig 4.6). To this end, we combined the cone-weight data (Fig 4.4) with information about each BC-terminal's stratification depth within the inner plexiform layer (IPL) (Fig 4.3C). In general, the IPL of all vertebrates studied to date is dominated by "Off-circuits" in the upper strata, adjacent to the somata of BCs and most amacrine cells, and by "On-circuits" in the lower strata, adjacent to the somata of retinal ganglion cells (Euler et al, 2014). Accordingly, light-components L_{tr} and L_{sus} are expected to mostly exhibit sign-conserving weights in the upper strata, and mostly sign-inverting weights in the lower strata (Fig 4.6A). Dark components D_{tr} and D_{sus} are expected to exhibit the reverse distribution (Fig 4.6B).

This textbook expectation, here graphically indicated by dashed lines, was indeed approximately met when considering dark-components (Fig 4.6B - note that UV-dark component weights were generally small and not further considered) and for light-components of UV-cones (Fig 4.6A, bottom panel). Similarly, this classical IPL organisation was also met by red-, green- and blue-cone weights for the upper two-thirds of the IPL, which included the traditional Off-layer, and the upper part of the traditional On-layer (Fig 4.6A, top three panels). However, specifically for red-, green- and blue-cones, the lower third of the traditional On-layer was dominated by weights of the "wrong" polarity (Fig 4.6A, top three panels). In agreement, most UV:R/G/B opponent clusters stratified in this lower third of the IPL (Figures 3C,4). Together, this suggests that several of these UV:R/G/B clusters are derived from sign-reversed red-/green-/blue-cone inputs onto "native" UV-On BCs, for example by way of amacrine cells.

Discussion

We have shown that the substantial spectral and temporal diversity of larval zebrafish BCs (Figs 3.1,3.2, cf. Zimmermann et al, 2018, Rosa et al, 2016) can be well-captured by a linear combination of inputs from the four spectral cone-types (Fig 4.3,4.4). This in turn allowed us to explore the major functional connectivity rules that govern spectral and temporal widefield signal integration by BCs: We find that red-cones overall provide the dominant input to BCs, often complemented by weaker but same-sign inputs from green- and blue-cones (Fig 4.5A,C,D). Likely as one consequence, BC pathways do not generally set-up new axes of spectral opponency in the mid- to long-wavelength range. Rather, they mostly either conserve and diversify the two major opponent motifs already present in the cones (Fig 4.5I,J) or establish non-opponent circuits (Fig 4.5F-H). In contrast, inner retinal UV-cone pathways appear to be organised essentially independently to those of red-, green- and blue-cones (Fig 4.5E). This leads to the consolidation of a third axis of spectral opponency, contrasting long- and mid-wavelength signals against UV (Fig 4.5K). This third axis appears to mainly stem from a systematic polarity reversal of inputs from red-, green- and blue-cones onto ‘natively-UV-On’ BCs in the lower IPL (Fig 4.6A).

Building spectrally opponent BCs. Because spectral opponency is a prominent feature in larval zebrafish cones (Yoshimatsu et al, 2021), BCs may inherit this property rather than set-up new opponent spectral axes by way of ACs. Indeed, the opponency observed in BC cluster C_{15} could be explained based on weighted but all-sign-conserving inputs from all four cones (Fig 4.4). However, the full picture may be more complex. For example, like C_{15} , cluster C_{14} was also opponent, albeit with a stronger long-wavelength response, and in this case the model used weakly sign-inverted red-cone weights alongside sign-conserved green- and blue-cone weights. In fact, most UV:R/G/B opponent clusters (e.g. C_{25-29}) required opposition of long versus short-wavelength cone inputs in the inner retina. This hints that inner retinal circuits may generally use a “mix-and-match” strategy to achieve diverse spectral responses by any available route, rather than strictly adhering to any one strategy. This notion is also tentatively supported by the presence of spectrally diverse amacrine cell circuits in adult zebrafish (Torvund et al, 2017). More generally, it perhaps remains puzzling how the complex interplay of cone pooling in the outer retina with AC inputs in the inner retina, across 29 highly diverse functional-BC-types which presumably express diverse receptors and ion channels (Euler et al, 2014), can ultimately be summarised in an functional wiring logic that for the most part simply sums all four cones, or ‘at best’

opposes a red-/green-/blue-system against UV. Resolving this conceptual conflict will likely require targeted circuit manipulations, for example by comparing BC spectral tunings in the presence and absence of amacrine cell inputs, or after targeted cone-type ablations.

Beyond ‘classical’ opponency, several clusters – both opponent and non-opponent – in addition encoded a notable mixture of spectral and temporal information. Interestingly, several of these clusters appeared to be concentrated around the centre of the IPL (e.g. C₂₀₋₂₅, Fig 4.2B,C) – a region which also in mammals has been associated with both transient and sustained processing (Franke et al, 2017, Baden et al, 2013, Roska & Weblin, 2001, Matsumoto et al, 2019). In zebrafish, a mixed time-colour code was previously described for the downstream retinal ganglion cells (Zhou et al, 2020), which now raises the question to what extent ganglion cells may inherit this property from BCs. Moreover, if and how such information can be differentially read out by downstream circuits and used to inform behaviour remains unknown.

Three axes of spectral opponency. In principle, the four spectral cone types of larval zebrafish could be functionally wired to for tetrachromatic vision. This would require that all four cone types contribute independently to colour vision. Theory predicts that efficient coding of colour should be based on four channels, an achromatic channel with no zero-crossings on the spectral axis, and three chromatic opponent channels with one, two and three zero-crossings respectively (Buchsbaum & Gottschalk, 1983, Atick & Redlich, 1992). However, such a coding strategy is not essential as demonstrated by the trichromatic visual system of many old-world monkeys which is based on two axes of opponency (“blue-yellow” and “red-green”), each with a single zero crossing. In the present study, we find that among zebrafish BCs, three zero-crossings predominate (Fig 4.5M,N, Fig 4.7A). Here, the single BC cluster with two zero-crossings (C₇) did not set-up any notable additional spectral crossings either, but instead crossed once in the ‘blue-cone position’, and once again near the ‘UV-red opponent position’ (Fig 4.5K). Nevertheless, our findings support the notion that at least at the level of BCs, and under the stimulus conditions used in this study, the zebrafish visual system is capable of supporting tetrachromatic colour vision, as observed behaviourally in goldfish (Neumeyer, 1992). If and how the larval zebrafish BCs’ axes are preserved, diversified, or even lost in downstream circuits will be important to explore in the future. In this regard, both retinal ganglion cells (Guggiana, et al, 2021, Zhou et al, 2020) and brain circuits (Guggiana et al, 2021, Fornetto et al, 2020) do carry diverse spectral signals, however beyond a global overview (Bartel et al, 2021) the nature and distribution of their spectral zero-crossings remain largely unexplored.

Links with mammalian SWS1:LWS opponency. Of the three spectral axes that dominate the zebrafish inner retina (Fig 4.5I-N, 7A), those functionally linked with green- (RH2) and blue-cone (SWS2) circuits are unlikely to have a direct counterpart in mammals where these cones-type are lost (Baden & Osorio, 2019, Baden, 2021). However, the third axis, formed by functional opposition of UV-cone circuits against red-/green-/blue-cone circuits, may relate to one or multiple of the well-studied mammalian SWS1:LWS opponent circuits (Neitz & Neitz, 2017, Marshak & Mills, 2013) (Fig 4.7B).

Despite substantial spectral variation amongst both SWS1 and LWS cone-types across species, mammals usually oppose the signals from SWS1-cones with those of LWS-cones at a retinal circuit level (Dacey, 2000, Dacey & Packer, 2003, Jacobs, 1993, Chen & Li, 2012, Mills et al, 2014, Szatko et al, 2020, Khani & Gollisch, 2021). For example, in the primate outer retina, SWS1-cones exhibit horizontal-cell mediated spectral opponency to LWS signals (Packer et al, 2010). Likewise, in the inner retina signals from a highly conserved SWS1-exclusive On-BCs are combined with those of LWS-biased Off-circuits in most if not all mammals that have been studied at this level (Behrens et al, 2016, Marshak & Mills, 2014, Breuninger et al, 2011, Dacey & Lee, 1994). Further such circuit motifs can involve diverse but specific types of amacrine and/or retinal ganglion cells (Dacey, 2000, Mills et al, 2014, Ghosh & Grunert, 1999).

Several of these mammalian motifs may have a direct counterpart in zebrafish. For example, like primate SWS1-cones, also zebrafish SWS1-cones exhibit weak but significant long-wavelength opponency that is mediated by horizontal cells (Yoshimatsu et al, 2021). Beyond this possible outer retinal connection, the inferred UV:R/G/B organisation in zebrafish BCs (Figures 5E,K, 6) is reminiscent of mammalian circuits associated with SWS1-BCs.

First, as in most mammals (Jacobs, 1993), SWS1_{On}:LWS_{Off} signals numerically dominate in zebrafish compared to SWS1_{Off}:LWS_{On} signals. Second, zebrafish SWS1:LWS opponent signals are predominately found in the lower-most (GCL-adjacent) fraction of the IPL (Figs 4.3, 4.6), the same place where mammalian SWS1-On BCs stratify (Behrens et al, 2016). Third, many zebrafish SWS1_{On}:LWS_{Off} signals occurred ventro-temporally (Fig 4.3D), the retinal region which in mice exhibits the highest density of type-9 BCs (Nadal-Nicolas et al, 2020), their only SWS1-exclusive BC type (Behrens et al, 2016, Breuninger et al, 2011). While zebrafish are not known to possess an SWS1-exclusive BC (Li et al, 2012), they do possess several anatomical BC types that contact SWS1-cones alongside either one or both of SWS2- (blue) and

RH2-cones (green) (Baden et al, 2021, Li et al, 2012). Such BCs may conceivably become SWS1-exclusive types upon the loss of RH2 and SWS2 cones in early mammalian ancestors.

However, not everything supports a direct correspondence between mammalian and zebrafish SWS1:LWS circuits. For example, in contrast to BCs, among the dendrites of the zebrafish retinal ganglion cells, most UV-opponent signals occur above the IPL midline, near the anatomical border between the traditional On- and Off-layers (Zhou et al, 2020). Nevertheless, this is approximately in line with the IPL position where several of the well-studied primate SWS1:LWS ganglion cells receive LWS-biased Off-inputs (Calkins et al, 1998), hinting that similar ganglion cell motifs might also exist in zebrafish. Certainly, zebrafish do possess a number of anatomical retinal ganglion cell types (Zhou et al, 2020, Robles et al, 2014) that display similar stratification patterns compared to those that carry SWS1:LWS opponent signals in diverse mammals (Marshak & Mills, 2014, Mills et al, 2014).

A summary of the above argument, showcasing possible links between retinal circuits for colour vision in cone-tetrachromatic species such as zebrafish, to those of most non-primate mammals and of old-world monkeys including humans, is suggested in Fig 4.7A-C. In the future it will be important to explore if and how mammalian circuits such as the ones carrying SWS1:LWS signals can be more directly linked with those found in zebrafish, for example by leveraging molecular markers across potentially homologous types of neurons (Shekhar et al, 2016, Peng et al, 2019, Kolsch et al, 2021).

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Author contributions

Conceptualization, PB, TY, TB; Methodology, PB, TY, FKJ; Investigation, PB, TY; Data Curation, PB, TB; Writing – Original Draft, TB; Writing – Review & Editing, TB, TY, FKJ; Visualization, TB, PB; Supervision, TB; Project Administration, TB; Funding Acquisition, TB.

Declaration of Interests

The authors declare no competing interests.

Star Methods

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tom Baden (t.baden@sussex.ac.uk).

Data and Code Availability. Pre-processed functional 2-photon imaging data and associated summary statistics is freely available on DataDryad under <https://doi.org/10.5061/dryad.wstqjq2n5> (Baden et al, 2021) and via the relevant links on <http://www.badenlab.org/resources> and <http://www.retinal-functomics.net>. See also [Data S4.1](#) for a graphical summary of key aspects pertaining to each BC cluster.

Materials Availability. The transgenic line Tg(1.8ctbp2:SyjGCaMP7bf) used in this study is available upon request to the lead author.

Experimental Model and Subject Details

Animals. All procedures were performed in accordance with the UK Animals (Scientific Procedures) act 1986 and approved by the animal welfare committee of the University of Sussex. Animals were housed under a standard 14:10 day/night rhythm and fed three times a day. Animals were grown in 0.1 mM 1-phenyl-2-thiourea (Sigma, P7629) from 1 *dpf* to prevent melanogenesis. For all experiments, we used 6-7 days post fertilization (*dpf*) zebrafish (*Danio rerio*) larvae.

Tg(1.8ctbp2:SyjGCaMP7bf) line was generated by injecting pBH-1.8ctbp2-SyjGCaMP7b-pA plasmid into single-cell stage eggs. Injected fish were out-crossed with wild-type fish to screen for founders. Positive progenies were raised to establish transgenic lines. The plasmid was made using the Gateway system (ThermoFisher, 12538120) with combinations of entry and destination plasmids as follows: pBH⁶⁷ and p5E-1.8ctbp, pME-SyjGCaMP7b, p3E-pA⁶⁸. Plasmid p5E-1.8ctbp was generated by inserting a polymerase chain reaction (PCR)-amplified -1.8ctbp fragment (Dreosti et al, 2009) into p5E plasmid and respectively. Plasmid pME-SyjGCaMP7b was generated by replacing GCaMP6f fragment with PCR-amplified jGCaMP7b (Dana et al, 2019) in pME-SyjGCaMP6f (Yoshimatsu et al, 2020) plasmid.

For 2-photon *in-vivo* imaging, zebrafish larvae were immobilised in 2% low melting point agarose (Fisher Scientific, BP1360-100), placed on a glass coverslip and submerged in fish water. Eye movements were prevented by injection of α -

bungarotoxin (1 nL of 2 mg/ml; Tocris, Cat: 2133) into the ocular muscles behind the eye.

Method Details

Light Stimulation. With fish mounted on their side with one eye facing upwards towards the objective, light stimulation was delivered as full-field flashes from a spectrally broad liquid waveguide with a low numerical aperture (NA 0.59, 77555 Newport), positioned next to the objective at $\sim 45^\circ$, as described previously (Yoshimatsu et al, 2021). To image different regions in the eye, the fish was rotated each time to best illuminate the relevant patch of photoreceptors given this stimulator-geometry. The other end of the waveguide was positioned behind a collimator-focussing lens complex (Thorlabs, ACL25416U-A, LD4103) which collected the light from a diffraction grating that was illuminated by 13 spectrally distinct light-emitting diodes (LEDs, details below). After mounting but before systematic light stimulation, fish were exposed to at least 5 minutes of “spectral noise” (each LED independently flickering in a random sequence) to light-adapt the eye.

An Arduino Due (Arduino) and LED driver (Adafruit TCL5947) were used to control and drive the LEDs, respectively. Each LED could be individually controlled, with brightness defined via 12-bit depth pulse-width-modulation (PWM). To time-separate scanning and stimulating epochs, a global “blanking” signal was used to switch off all LEDs during 2P scanning but enable them during the retrace, at line-rate of 1 kHz (see also Euler et al, 2019, Zimmermann et al, 2020). The stimulator code is available at <https://github.com/BadenLab/HyperspectralStimulator>.

LEDs used were: Multicomp Pro: MCL053RHC, Newark: C503B-RAN-CZ0C0AA1, Roithner: B5-435-30S, Broadcom: HLMP-EL1G-130DD, Roithner: LED-545-01, TT Electronics: OVLGC0C6B9, Roithner: LED-490-06, Newark: SSL-LX5093USBC, Roithner: LED450-03, VL430-5-1, LED405-03V, VL380-5-15, XSL-360-5E. Effective LED peak spectra as measured at the sample plane were, respectively (in nm): 655, 635, 622, 592, 550, 516, 501, 464, 448, 427, 407, 381, 360 nm. Their maximal power outputs were, respectively (in μW): 1.31, 1.06, 0.96, 0.62, 1.26, 3.43, 1.47, 0.44, 3.67, 0.91, 0.24, 0.23, 0.20. From here, the first ten LEDs (655 – 427 nm) were adjusted to 0.44 μW , while the three UV-range LEDs were set to a reduced power of 0.2 μW . This relative power reduction in the UV-range was used as a compromise between presenting similar power stimulation across all LEDs, while at the same time ameliorating response-saturation in the UV-range as a result of the UV-cones’

disproportionately high light sensitivity (Zhou et al, 2020, Yoshimatu et al, 2020). The same strategy was used previously to record from cones (Yoshimatu et al, 2021).

2-photon calcium imaging. All 2-photon (2P) imaging was performed on a MOM-type 2P microscope (designed by W. Denk, MPI, Martinsried; purchased through Sutter Instruments/Science Products) equipped with a mode-locked Ti:Sapphire laser (Chameleon Vision-S, Coherent) tuned to 927 nm for SyGCaMP7b imaging. Notably, like all calcium imaging, the biosensor exhibits non-instantaneous binding and unbinding kinetics, which in effect low-pass filters the “real” calcium signals in BCs. We used one fluorescence detection channel (F48x573, 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 6.3 for Windows (Wavemetrics).

All data was collected using a quasi-simultaneous triplane approach by leveraging an electrically tunable lens (ETL, EL-16-40-TC-20D, Optotune) positioned prior to the scan-mirrors. Rapid axial-jumps of $\sim 15\ \mu\text{m}$ between scan planes (ETL settling time of $< 2\ \text{ms}$ (Janiak et al, 2019)) were enabled by using a non-telecentric (nTC) optical configuration (nTC₁, 1.2 mm – see Janiak et al, 2019). This nTC optical setup is described in detail elsewhere (Janiak et al, 2019). All recordings were taken at 128×64 pixels/plane at 3 planes (5.2 Hz effective “volume” rate at 1 ms per scan line).

Pre-processing of 2-photon data, IPL detection and ROI placement. Raw fluorescence stacks were exported into a Python 3 (Anaconda) environment. The data were de-interleaved and separated into the three recording planes. Next, the data were linearly detrended, linearly interpolated to 42 Hz, and aligned in time. The anatomical borders of the inner plexiform layers were automatically detected by first median-smoothing the time standard deviation images with a Gaussian kernel size of 3 pixels. From here, every pixel above the 35% per-image amplitude threshold was registered as IPL. This automated procedure was made possible by the fact that GCaMP6f expression was restricted to the presynaptic terminals of BCs, which also defined the anatomical borders of the IPL.

To place regions of interest (ROI), a quality index (QI) as described previously (Baden et al, 2016) was calculated for each pixel. In short, the QI measures the ratio of variance shared between stimulus repetitions and within a single stimulus repetition. The larger the QI, the more variance in the trace is due to the presented stimulus:

$$QI = \frac{VarC_{r_t}}{VarC_{t_r}}$$

where C is the T by R response matrix (time samples by stimulus repetitions) and $\bar{x}$ and $Var[x]$ denote the mean and variance across the indicated dimension, respectively. QI ranges from 0 (perfectly random) to 1 (all stimulus repetition responses are identical). This yielded “QI-images” that indicated where in a scan BC-responses were located. From here, ROIs were automatically placed using custom Python scikit-image scripts⁷³. In brief, QI-images were adaptively thresholded using kernel size 5 pixels which helped accentuate responsive image structures that were approximately BC-terminal-sized (in our scan configuration, most BC-terminals were ~5 pixels in diameter – cf. Fig 4.1D). The resulting binary images were distance-transformed and shrunk. The contours of the remaining groups of pixels were recorded and filled, and the highlighted pixels were used as ROI coordinates. This yielded ROI sizes of $1.36 \pm 0.17 \mu m^2$ (mean $\pm$ SD), which is in line with anatomical sizes of BC terminals in larval zebrafish (Baden et al, 2014). While it remains possible that a minority of ROIs over- or under-split terminals, this possible limitation was judged to be minimal based on manual inspections. The IPL position of each ROI was defined as the relative position of the centre-of-mass of the filled ROI contour to the nearest inner and outer borders of the IPL.

ROI traces were converted to z-scores. For this, a 5 s portion of the trace preceding stimulus presentation was drawn and defined as baseline. The standard deviation of this baseline fluorescence signal was calculated and used to z-score the remainder of the trace. Finally, QIs as described above for each pixel were also calculated for each ROI. In line with how we previously processed the cones (Yoshimatsu et al, 2021), ROIs with $QI < 0.4$ were excluded from further analysis. $n = 6,125$ ROIs passed this quality criterion (72 triplane scans from 7 fish).

Clustering of BCs. To identify structure amongst the BC-dataset, trial-averaged ROI traces were PCA-transformed and clustered as described previously (e.g. Zimmermann et al, 2018, Baden et al, 2016). In brief, we used the first 48 principal components, which accounted for 82% of total variance. Of these, components that near-exclusively carried high-frequency content which is likely linked to noise were discarded. The transformed time-traces were clustered using the scikit-learn (Python 3, Anaconda) implementation of the Gaussian Mixture Models algorithm. The number of clusters (29) was determined using the Bayesian information criterion (BIC). However, the BIC curve notably flattened above ~20 clusters, suggesting that a range of solutions would be

similarly plausible. Clusters were judged as stable over repeated clustering runs starting from different random seeds, in the sense that they always picked up several broadband and UV:R/G/B response types, followed by a smaller number of “cone-like” ones (cf. Figure. 4.5).

Reconstruction of BC responses from cones.

To reconstruct each BC-mean response into constituent spectral and temporal components, we combined the average spectral tuning curve of each of the four cone-types (from Yoshimatsu et al, 2021) with four temporal components associated with a given light response (i.e. 1.5 s On, 1.5 s Off). The four temporal components used, obtained by non-negative matrix factorisation across all light responses and cluster means, resembled light-transient, light-sustained, dark-transient, and dark-sustained temporal profiles (Fig 4.3B). Next, each ROI's trial averaged trace was decomposed into a corresponding 4 by 10 array (four temporal components X 10 LEDs). Here, we restricted the reconstruction to the central 10 LEDs that generally elicited the greatest variance across BCs. This also avoided using responses to the shortest wavelength LED which may have driven saturating responses in UV-cones (UV-cones are more light-sensitive than the other cones). Moreover, it avoided using the two longest-wavelength LEDs where responses were comparatively weak and thus noisy.

This yielded four spectral tuning curves per ROI (i.e. light-transient x 10 LEDs, light-sustained x 10 LEDs and so on), which were then linearly interpolated to the range of 360 - 610 nm to conform with the cone data format. The BC tuning curves were then modelled as linear combinations of the cone tuning curves with a lasso regulariser, which yielded four cone weights X four response bases per BC-trace. For simplicity, we henceforth used the ROI-averaged weights within a cluster for further processing, but each ROI's individual weights are available to download from DataDryad (Baden et al, 2021).

To assess reconstruction quality (Fig S4.2), reconstructed data was subtracted from the original ROI-means to yield residuals. From here, we compared original data, reconstructions, and residuals by two metrics: variance explained across all clusters, and temporal power explained. To determine the fraction of variance explained by the reconstructions, we first computed the total variance across all clusters for each time-point. The result of this process, plotted beneath each corresponding heatmap (Fig S4.2A), showed similar time-variance profiles across cluster means and their reconstructions (panels 1 and 2), but very little remaining signal for the residuals (panel 3). From here, we computed the area under the curve for each variance-trace and

normalised each to the result from the original cluster means. By this metric, cluster reconstructions captured 94.0% of the original variance, while residuals carried 5.1%.

To determine the extent to which temporal structure was captured, we used a similar approach to the one above, however in this case based on a magnitude-squared Fourier Transform of each time-trace (Fig S4.2B), limiting the result between 0.16 and 2 Hz which captured the bulk of physiologically meaningful temporal components given the optical imaging approach used (i.e. lower-frequency components would mainly arise from imperfect detrending, while higher-frequency components would exceed the Nyquist recording limit, and further be limited by the kinetics of GCaMP7b. From here, we computed the average of all clusters' Fourier transforms (plotted beneath each panel) and again computed the fraction of this signal captured by the reconstruction (103.8%) and residuals (3.8%). Notably, while this metric was mainly informative about low frequency components which dominated all signals, also higher frequency components were generally well captured, as visible in the individual heatmaps.

Quantification and Statistical Analysis

Statistics. No statistical methods were used to predetermine sample size. Owing to the exploratory nature of our study, we did not use randomization or blinding. To compare weight amplitude distributions (Fig 4.5A,B) we used the paired Wilcoxon Rank Sum Test, taking paired components as the input (i.e. comparing red-light-transient versus green-light-transient, and so on). To assess weight correlations between cones (Fig 4.5C-E, Fig S4.2), we in each case list the Pearson correlation coefficient ρ and 95% confidence intervals (CI) based on the mean weights per cluster. Individual temporal weights were not considered in this analysis. All statistical analysis was performed in Python 3 (Anaconda) and/or Igor Pro 6 (Wavemetrics).

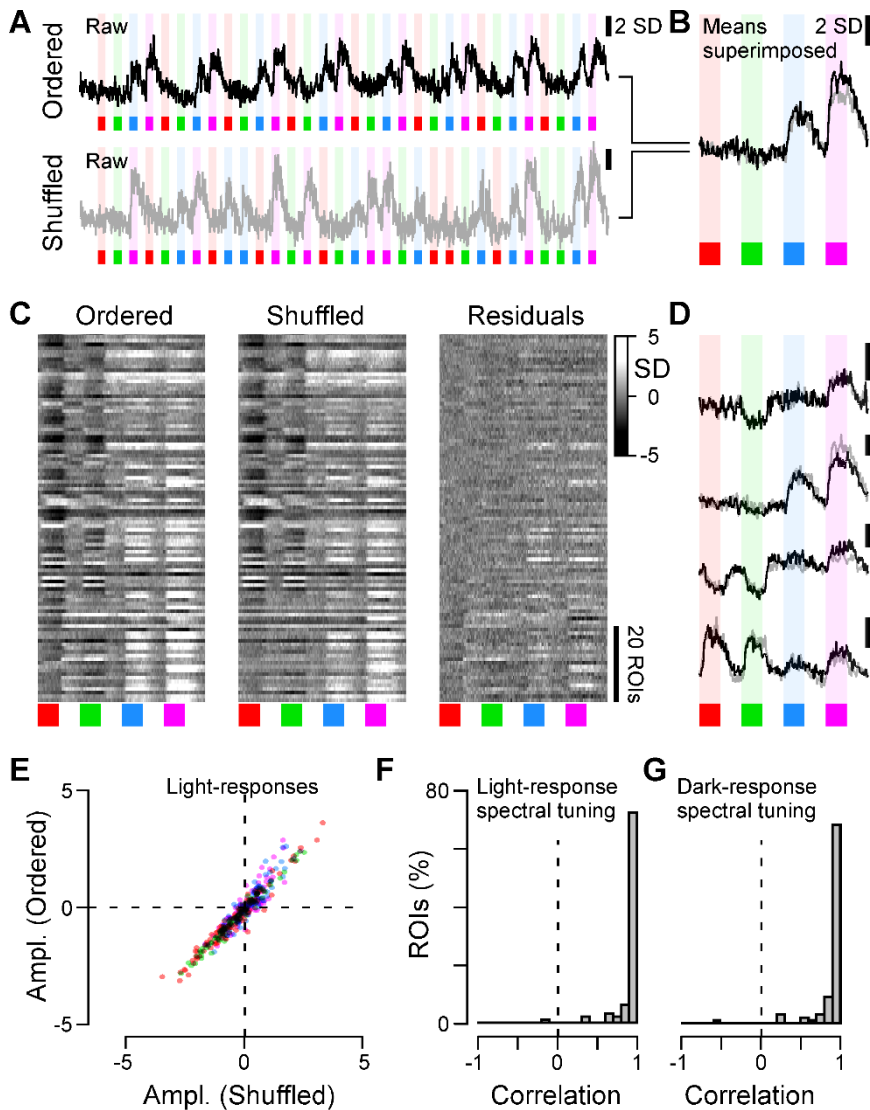


Fig S4.1: Detailed Summary for each Cluster.

Related to Fig 4.4. For each of the 29 clusters as indicated (1 cluster per page), overview of key response aspects and analysis as shown across the main figures. Upper row, from top: Stimulus sequence, heatmap of the response-mean of all ROIs assigned to the cluster (as in Fig 4.2A), cluster mean $\pm$ SD (grey, as Fig 4.2B) and reconstruction (black, as Fig 4.4A). Lower row, from top/left: Allocated cone-weights (as Fig 4.4B), here with SD error bars across each individual ROI that contributes to a cluster, and bottom left: distribution of ROIs across the IPL and eye (as Fig 4.2C,D). Bottom right: Spectral tuning functions extracted from cone weights for each temporal component as indicated (thin lines, as Fig S4.3F,G) and bulk tuning function based on the combination of all temporal components (thick line, as Fig 4.5F-K).

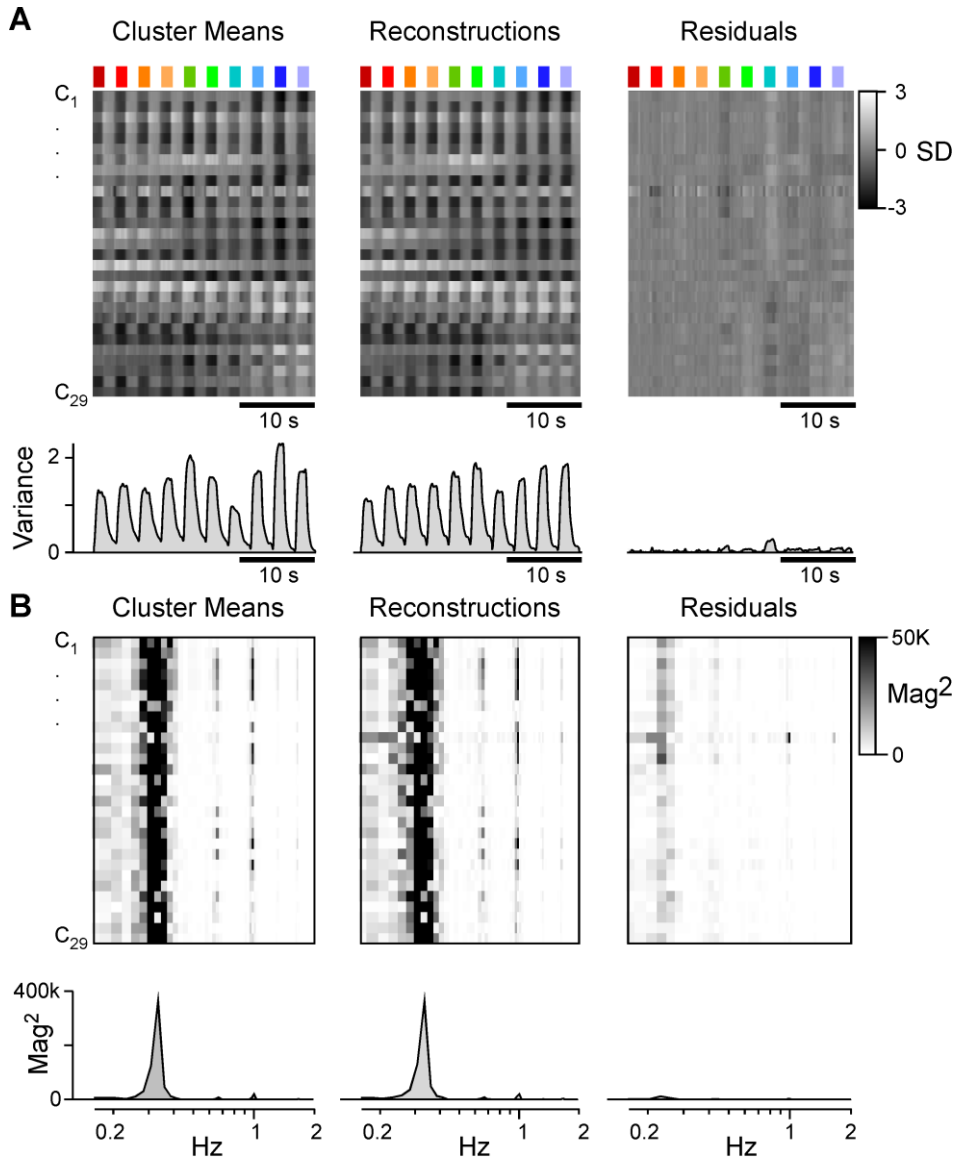


Fig S4.2: Cluster reconstruction details.

Related to Figure 4.3. A, Time-aligned heatmaps of all cluster means (left) are shown alongside their corresponding reconstructions (middle) and residuals (right). The time trace below each cluster shows the total variance across all clusters per time point (Methods). B, as A, but for magnitude-squared Fourier transforms of each cluster, reconstruction, and residuals. The traces below each panel show the averages of these transforms across all clusters (Methods). Note that for both (A) and (B), residuals retain only a small fraction of the original signal, indicating high reconstruction fidelity. Reconstruction quality of each individual cluster can further be assessed in Appendix 1

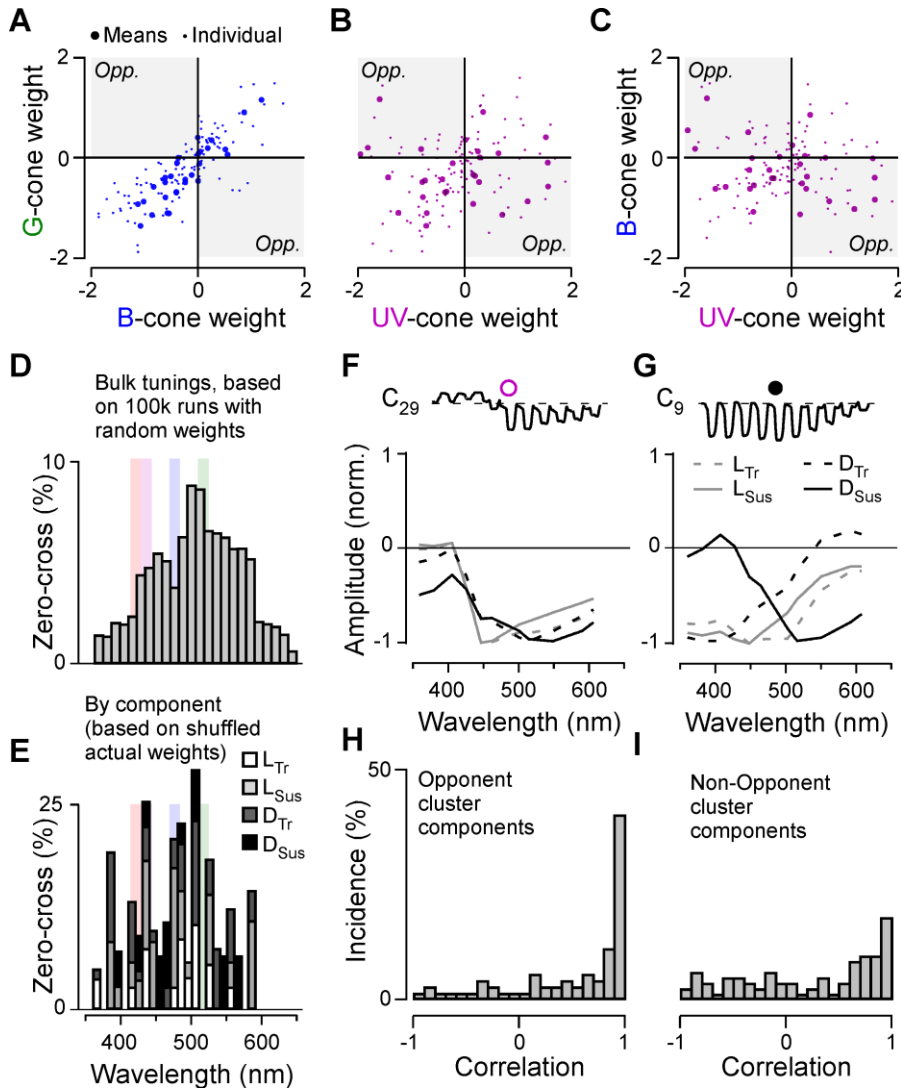


Fig S4.3: Spectral tunings and temporal components.

Related to Figure 4.5. A-C, As Figure 4.5C-E, but showing weight correspondences between green-blue, green-UV and blueUV cones, respectively. D, As Figure 4.5M, but following based on 100,000 iterations using randomised values (between -5 and 5) for each of the 16 weight variables. E, as Fig 4.5N, but following random permutation of time-components across cones. F,G, Spectral tuning functions for two example clusters (C₂₉ and C₉, respectively), computed individually by temporal components as indicated. Note that for C₂₉ (F), the four tuning functions were similar to each other, while for C₉, the tuning of the dark-sustained component deviated strongly from that of the remaining three components. Corresponding time-component resolved tuning functions are detailed for each cluster in Appendix 1. H,I, Distribution of correlations between each cluster's "time-component spectral tuning functions" as illustrated in (F,G), for spectrally opponent clusters (H), and for non-opponent clusters (I).

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
none		
Chemicals, Peptides, and Recombinant Proteins		
α -bungarotoxin	Tocris	2133
Agarose low melting	FisherScientific	BP1360-100
Deposited Data		
All preprocessed data including each ROI's trial-averaged response and metadata (size, position etc) as well as each ROI's 16 weights	This paper, DataDryad	https://datadryad.org/stash/dataset/doi:10.5061/dryad.wstqjq2n5
Experimental Models: Organisms/Strains		
Danio rerio (zebrafish): <i>Tg(1.8ctbp2:SyjGCaMP7b)</i>	This paper	
Recombinant DNA		
pBH-1.8ctbp2-SyjGCaMP7b-pA	This paper	
pBH	[67]	
P5E-1.8ctbp	[68]	
pME-SyjGCaMP7b	[68]	
p3E-pA	[68]	
p5E-1.8ctbp	[31] and this paper	
pME-SyjGCaMP7b	[69], [70], and this paper	

Software and Algorithms		
Python 3 (Anaconda)		
Igor Pro 6	Wavemetrics	

5. Tectal Processes and Brain Somatic Data: uniformity of spectral sensitivity

*He'd extract numbers out of matter,
And keep them in a glass, like water,
Of sov'reign power to make men wise:
For, dropt in blear, thick-sighted, eyes,
They'd make them see in darkest night,
Like owls, tho' purblind in the light.*
Samuel Butler, *Hudibras*, Part 1, Canto 1

Introduction

In this chapter I show preliminary analysis of spectral sensitivity profiles obtained from the zebrafish tectum and zebrafish brain neurons. Ultimately, to guide behaviour, signals processed in the cones and bipolar cells of the retina must reach the brain. What signals does the brain receive?

The tectum is the principal retinorecipient part of the brain in zebrafish. It is located posteriorly and dorsally to the eyes. It is comprised of the neuropil as well as both local and broadly projecting neurons (e.g. Wulliman, 2012, Niell & Smith, 2005).

Retinal Ganglion Cells (RGCs) send their axons through the optic nerve primarily to the contralateral tectum. There is a degree of decussation, with some afferents sent to the ipsilateral tectum. Many RGCs arborise both in the tectum itself (AF10), but send collaterals to small areas (arborization fields, AF 1-9) outside of the tectum along the path to their primary target (tectum) (Robles et al. 2014, Kramer et al, 2019). A small number of RGCs do not project to the tectum, but just beneath to innervate the largest-nontectal AF (AF9). AFs 1-9 are generally associated with rather specific behaviours (e.g. AF7 is associated with prey capture (Semmelhack et al, 2014)), while AF10 is often thought of as a more general processing centre of vision (Niell & Smith, 2005).

We assume that neural structures situated closely are more likely to have functional connections. Axons that terminate next to each other are more likely to terminate and affect the same postsynaptic structures. The import of this assumption is highlighted in e.g. work by Li et al (1990s). Hence anisotropy with respect to some stimulus dimension is interesting because it is intelligible. E.g. some form of chromatic gradient (lamination) in the tectum will appear to be chromatic processing.

The tectum itself is retinotopically organised (Northmore, 2011) along its 2 principal axes, and perpendicularly to this retinotopic plane it is composed of multiple layers with distinct RGCs inputs, local processing circuits, and brain projections. Responses to different stimuli may relate to tectal neuropil organisation differently. As an example, responses to motion obviate laminae in the tectum (Niell & Smith, 2005). For example, the retinotopic representation of motion may not match the spectral sensitivity organisation. Indeed, there is no *a priori* reason for it to do so. To this notion, cortical structures in different species exhibit different columnar organisation with respect to motion direction, orientation and chromatic axes (Goodhill & Carreira-Perpiñán, 2006).

Ultimately, it is not the eye, but some **brain** region or neuron that directly represents and is the most proximal cause of motor commands (in the sense outlined in **Ch1**). Higher visual areas are hypothesised to have neurons with highly selective receptive fields, the hypothesised “Grandmother cell”, as an example (Poggio, 1990). Such hypothesised RFs may conditionally bind a particular feature of the visual world with a particular behaviour. In animals with a limited behavioural repertoire one expects to find such neurons more readily. Zebrafish is an example of such an animal. Therefore, in addition to surveying RGC axonal projections, I also wanted to explore the types of chromatic responses of brain neuron somata in general.

To investigate spectral sensitivity profiles in the tectum and the brain, I carried out large-scale recordings during the presentation of the described Sweep Stimulus using the Light Synthesiser. I will first describe the dataset from RGC processes, and later present the data from brain somata.

RGC responses in the brain

The tectum is a highly organised, 3D structure that presents visual space along 2 axes, and distinct functions in layers along the third. The tectum in the larval zebrafish is inclined at ~ 30 deg relative to the lateral-medial plane (Wulliman, 2012), meaning that a perfectly horizontal (or vertical) optical recording plane will cut the tectum at an angle relative to its native organisation. To address this issue we modified the 2-photon microscope to allow recording from “tilted” planes. We introduced a second recording plane (Anterior-Posterior), nearly-perpendicular to the first one. This allowed me to observe multiple lamination axes. It is difficult to assess the precise positioning of the planes relative to the fish until image registration takes place.

Specifically, the ETL setup devised within the laboratory (Janiak et al, 2019) was employed, albeit in a different fashion. ETL dioptries are modulated on every line; allowing us to sculpt the incline of the recording plane. This enabled recording tectal activity from two non-parallel recording planes – one cutting the tectum across its layers at a fixed retinotopic axis, and the other cutting the tectum along its upper layer(s) across all of retinotopic space (Fig 5.1).

Neuronal responses in the tectum and the brain are qualitatively different to responses in upstream neuronal layers. PRs and BCs produce gradual responses, otherwise known as analog responses. RGCs and many downstream neurons communicate with action potentials. This is known as the Analog-to-Digital switch (e.g. Zbili & Debanne, 2019). Ca^{2+} responses are expected to be different between the “Analog” and the

“Digital” populations. In the digitised data we expect to see rectification of the Ca^{2+} responses.

Isl2b – Ganglion cell axonal arborisations in the brain

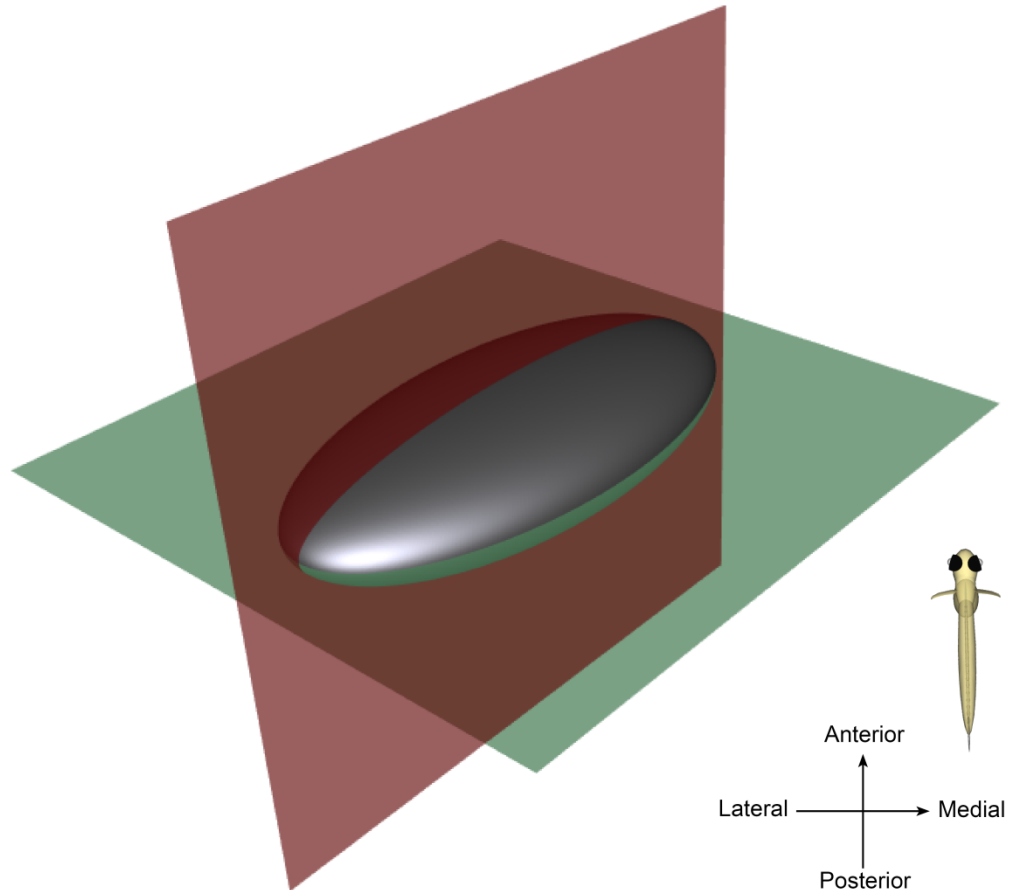


Fig 5.1 Recording planes in the tectum.

The tectum is schematically represented in gray. Two principal planes of recording are in red and green, respectively.

Collected data show remarkably uniform responses in the tectum. This is a stark contrast to Bipolar Cell spectral response profiles.

The analysis presented here is **preliminary**. Ideally, neuropil data should be partitioned into coinciding structural and functional units, comparative to cells. Processes do not have computationally beneficial shapes, however. Also, signal from different processes can co-localise, and that would require signal demixing. Up to now, neuropil segmentation is an unsolved problem. Developers of CalmAn (Giovanucci et al, 2019) show promising results; but I was unable to achieve structural segmentation with their

suite.

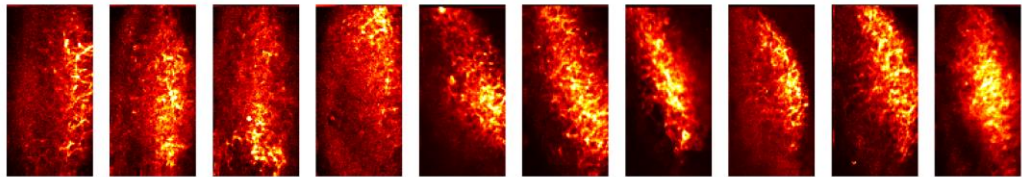
Registration to anatomical atlases is a staple in such investigations. Unfortunately, physical facilities did not allow for this, though I tried. I nevertheless believe that I captured the major trends in the dataset in the following analysis.

Animals and Tissue Preparation, Choice of Age of Zebrafish Larvae are nearly identical to those from **Ch4**, but also see **Ch2**. The following are the differences:

The fish lines used are *isl2b-SyGCaMP6f* (Johnston et al, 2019) and *H2B:GCaMP6f* (Dunn et al, 2016) for tectal and neuronal data, respectively. To robustly observe different response components, we doubled the length of the stimulus to present 3 s flashes interleaved with 3 s gaps (rather than 1.5 and 1.5 s).

Tectal Recordings, SD Projections

Medial-Lateral Plane, n(fish)=4



Anterior-Posterior Plane, n(fish)=6

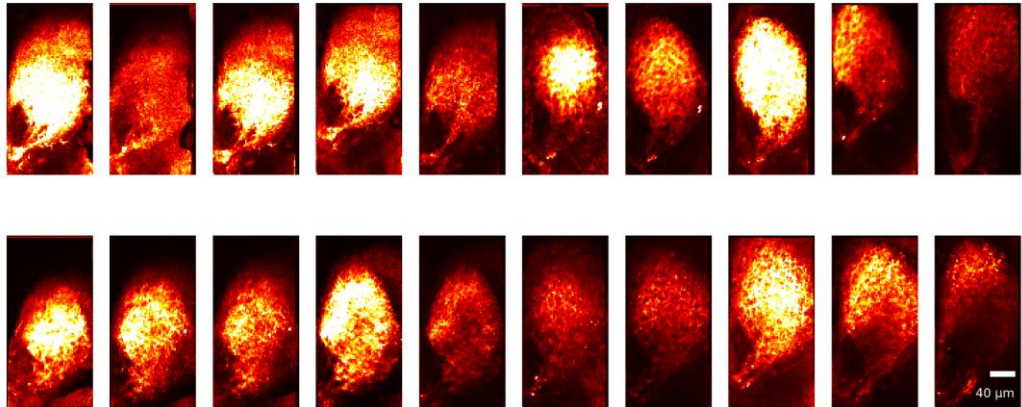


Fig 5.2 Example recordings from the medial-lateral and the anterior-posterior planes.

Presented is the part of the dataset for which the analysis is written below. Additional data were from the pretectal areas (data and analysis not presented).

Data Analysis

Fluorescence data are collected using the Igor Imaging Software (for details see **Ch2**).

They are exported into a Python 3 (Anaconda) environment. The data are de-interleaved and separated into different recording planes. The data are linearly detrended; linearly interpolated to ~42 Hz; and aligned in time. After that, only the data obtained during stimulus presentation are stored. Quality Index (as described in Baden et al 2016) is calculated for individual pixels as described in **Ch4**.

Data Alignment

The recording planes are aligned as follows: The recordings were manually divided into two groups, one from each recording plane alignment), showing significantly different structures (see Fig 5.2). The recordings are averaged in time. The averaged images are rotated until an angle with the highest amplitude projection was found. Heuristically; due to the nature of the tectal calcium response, this provides a good rotation angle to align to. The Images are then convolved with a reference image to find a robust shift that maximised the convolution. The alignments are then manually checked by me.

Principal Component Analysis

Pixels do not represent single structures. Hence, non-interpretable variance (“noise”) is present at this analysis level. To de-noise the data, I transform them using Principal Component Analysis.

The preprocessed data are averaged over stimulus loops and stacked across recordings such that time-aligned Ca^{2+} -response traces were rows of the resulting matrix. The matrix is dimension-reduced using the *sklearn* implementation of the PCA algorithm. The first 22 Principal Components account for 90% of the variance in the data.

Non-negative Matrix Factorisation

I decided to cluster the pixel responses. Further plans include fitting the response curves with either cone responses or BC terminal responses. To that end I further decomposed response waveforms into constituent non-negative components.

Data are PCA inverse-transformed and reshaped to [Length of Response to LED x (Number of Pixels per Scan x Number of Scans)]; so that rows of the matrix are filled with Ca^{2+} -responses to individual LED presentation. 12000 rows from the resulting matrix are randomly drawn. The *sklearn* implementation of the Non-negative Matrix Factorisation (NMF) algorithm was used to produce interpretable response waveform bases. Each trace was thus represented by a vector of length 17 (LEDs) X 4 (Non-negative components).

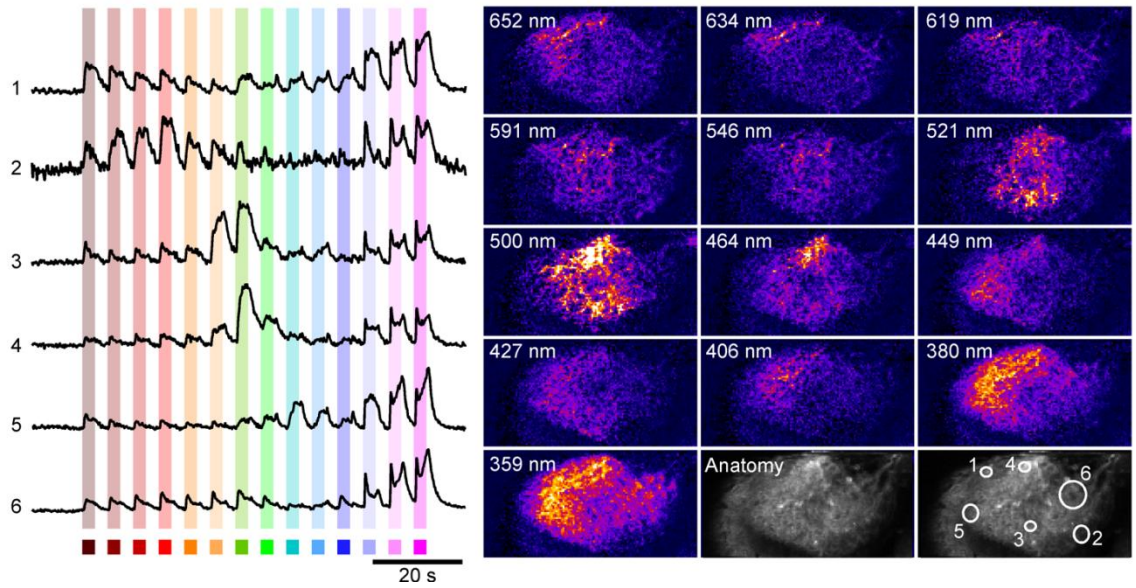


Fig 5.3 Example response traces from different tectal areas.

There is considerable regional diversity of spectral response.

Left: Average trace examples from the areas highlighted on the bottom right, z-scores.

Right: Spatial footprints of responses at stimulus onsets of the noted wavelengths.

Clustering

The resulting vectors are z-normalised and clustered using the Sklearn-implementation of the Gaussian Mixture Model (GMM) clustering algorithm. This resulted in over 40 functional clusters. Vast majority did not show spatial structure. The clusters are discarded if the allotted traces fail the QI criterion (see **Ch4 methods** for details) at 0.4 threshold. All such clusters did not show spatial structure.

Results of the clustering can be seen in Fig 5.4.

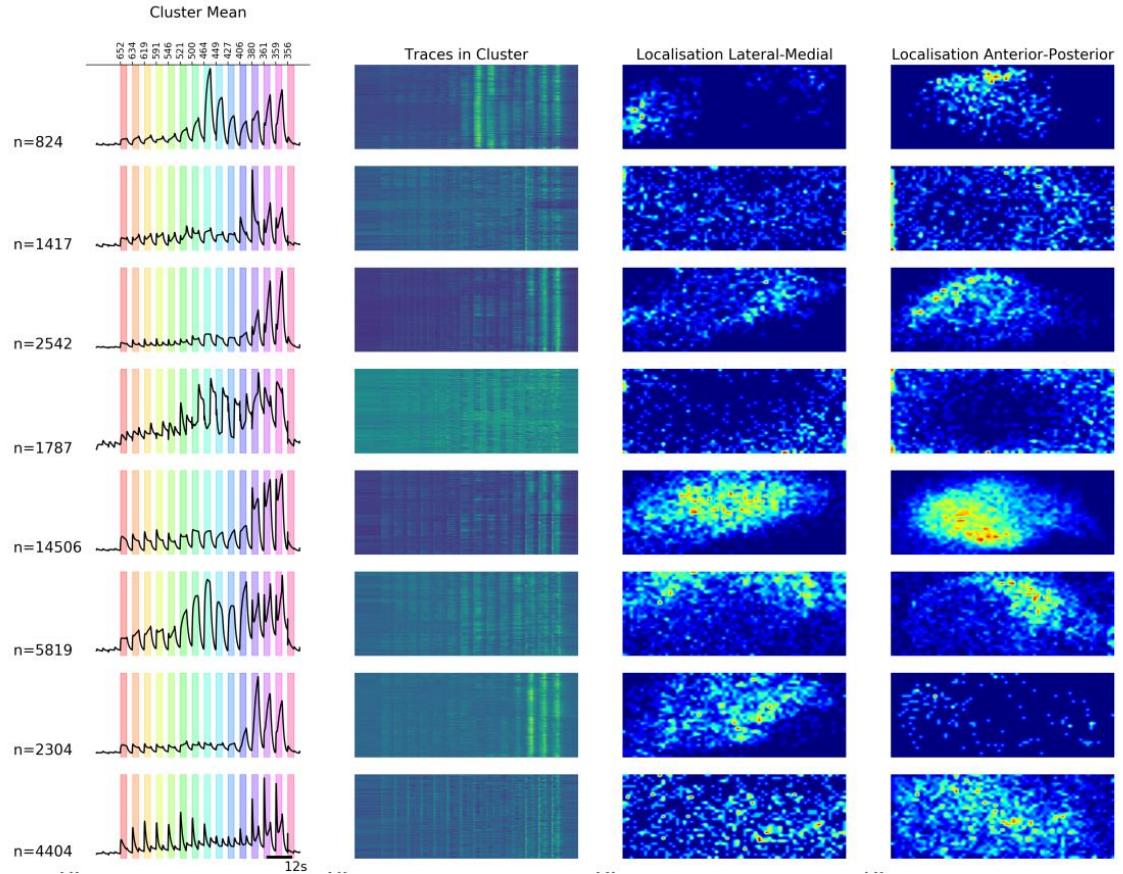


Fig 5.4 Pixel response clusters.

Left to right:

Cluster centroids, z-scores.

All traces within the cluster

2d-histogram of pixel positions within the cluster; projected along the lateral-medial recording plane.

2d-histogram of pixel positions within the cluster; projected along the anterior-posterior recording plane.

Nearly all pixels are broadband, with most prominent responses to UV light. Nearly all pixels respond to stimulation with an ON increase in Ca^{2+} -fluorescence. Responses differ in their transience and spectral response peak. A pixel is usually consistently sustained or transient with the exception of its UV response (see clusters 5-6 as an example). C1 presents a small ($n=824$) collection of pixels that are more mid-wavelength-responsive. The vast majority (C 5, 7) is strongly UV-biased

C1 shows strong localisation both in the L-M and the A-P planes. There are signs of wavelength-dependent lamination in the A-P and L-M axes: consider the localisation and the response profile of clusters 5 and 6. Overall, it seems that **the edges of the tectal neuropil process more diverse chromatic information than does the central neuropil.**

The clusters do not reflect underlying anatomy.

In the previous section I superimposed different cluster labels on the time-averaged recording videos. The resulting images show spatial structure. These appearances are not to be confused with anatomical structures. I show spatial principal components of an example recording in Fig 5.5.

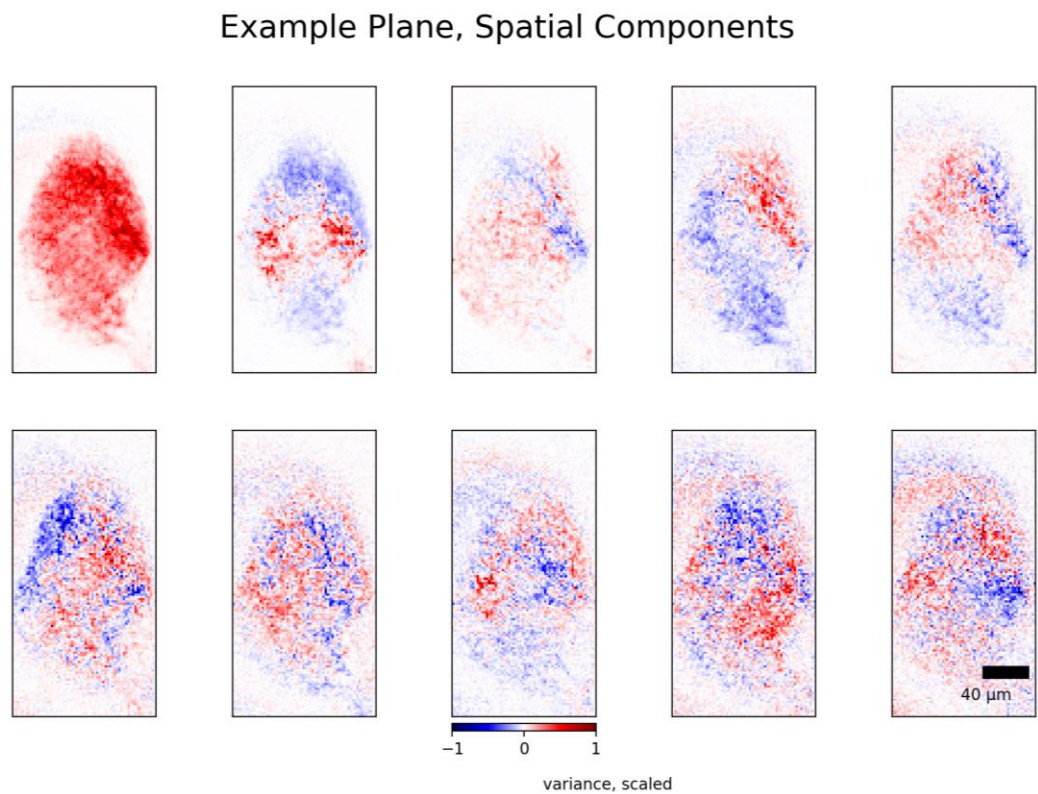


Fig 5.5 Spatial PCA reveals structures.

10 first spatial PCs presented left to right, top to bottom, in order of proportion of variance explained.

The spatial components show spatial structure. The spatial components are not identical to label footprints of different clusters. Hence, two procedures suggest different anatomical structures. There is no a priori reason why any one of them should reflect anatomical structures veridically.

Spatial PCs reflect anatomical structure better than Temporal ones because the same underlying anatomical structure fires together reliably in space. Assignment of a cluster label does not permit overlaps between clusters. Principal components can overlap in space, just like real neuronal processes do. Hence, **it is likely though not necessary that the functional clusters best represent mixtures of signals from different colocalized anatomical structures**. If pixels represent axon portions terminating proximally to each other; their tuning curves will be more similar to the consequent neural layer's; than if they represented anatomical structures.

Discussion

Strikingly, the diversity of responses present in the BC terminals is largely lost. Tectal responses are similar in response profiles and **do not exhibit noticeable opponency**.

I found nearly ubiquitous UV-responses in the tectum. Other wavelengths are sparsely represented in the tectal neuropil. These results cast a new light on the idea of "Optic Tectum". It is thought that only the most dorsal tectal layers are visual areas. I attribute it to the fact that most researchers use "amber light" (ca. 580 nm, e.g., Johnston et al, 2019). The results appear to show both functional lamination in with respect to wavelength sensitivity in the tectum; and ubiquitously visual nature of the tectal neuropil. Indeed, GC axon terminals are found throughout the tectal neuropil. Hence, previous functional findings are brought in accord with the anatomical tracing (Robles et al, 2014).

I am likely missing most the complexity present in tectal responses. Indeed, RFs in the tectum proper tend to be small and highly selective (Wang et al, 2020).

This portion of the chapter was published in Current Biology. Here formatted for readability.

Colourfulness as a possible measure of object proximity in the larval zebrafish brain

Philipp Bartel^{1§}, Filip K Janiak¹, Daniel Osorio¹ and Tom Baden^{1,2}

The encoding of light increments and decrements by separate On- and Off- systems is a fundamental ingredient of vision, which supports edge detection and makes efficient use of the limited dynamic range of visual neurons (Westheimer, 2007). Theory predicts that the neural representation of On- and Off-signals should be balanced, including across an animals' visible spectrum. Here we find that larval zebrafish violate this textbook expectation: in the zebrafish brain, UV-stimulation near exclusively gives On-responses, blue/green stimulation mostly Off-responses, and red-light alone elicits approximately balanced On- and Off-responses (see also Zhou et al, 2020. Guggiana et al, 2021, Fornetto et al, 2020). We link these findings to zebrafish visual ecology, and suggest that the observed spectral tuning boosts the encoding of object 'colourfulness', which correlates with object proximity in their underwater world⁵.

To begin, we measured high-acuity spectral sensitivities of larval zebrafish brain neurons by two-photon imaging, capturing $n = 11,967$ Regions-Of-Interest (ROIs) across the brains of $n = 13$ six to seven day post-fertilization zebrafish (elavl3:H2B-GCaMP6f; Fig 5.6A, Fig S5.1A–C in the Supplemental Information). To record the entire brain along its natural three-dimensional curvature we used a non-telecentric mesoscale approach coupled with 'intelligent plane bending' enabled by rapid remote focusing (Janiak et al, 2019) (Video S5.1, Fig S5.6A). A custom hyperspectral stimulator consisting of 13 spectrally distinct LEDs opposing a diffraction grating and collimator for collection (Yoshimatsu et al, 2020) allowed wide-field stimulation, which was approximately aligned with one eye's retinal acute zone. Regions of interest corresponding to individual and/or small groups of similarly responding neuronal

somata were extracted from each recording, then quality filtered, denoised and decomposed into On- and Off- responses (Fig S5.1A–G, Supplemental Experimental Procedures).

Recordings revealed that, despite some expected variation (Zhou et al, 2020, Guggiana et al, 2021, Fornetto et al, 2020) (for example, Fig S5.1B), neural responses in all major visual centres of the brain had a common, overarching spectral sensitivity profile: UV-On, Blue/Green Off, Red On-Off (Fig 5.6B). This organisation into three spectral processing zones (UV, Blue/Green, Red) can be linked to visual ecology. First, the UV On- responses likely serve prey-capture of aquatic microorganisms such as paramecia, which appear as UV-bright objects when illuminated by the sun (Yoshimatsu et al, 2020). Second, the approximate balance of red On- and Off- responses may allow zebrafish to use the abundance of long-wavelength illumination in shallow water (Zimmermann et al, 2018) to drive ‘general-purpose’ achromatic vision, including motion circuits (Orger & Baier, 2005). Third, the dominance of Off responses to blue and green wavelengths may serve as a subtraction signal to spectrally delineate the red- and UV-systems (Zhou et al, 2020), and to provide a spectral opponent signal for colour vision against UV- and red-On circuits (Yoshimatsu et al, 2020).

A further non-mutually exclusive interpretation is that spectral organization in zebrafish brain accentuates ‘colourfulness’, which could act as a cue to object proximity. This is because unlike air, turbidity in aquatic environments rapidly attenuates both achromatic and chromatic contrasts with distance (Wilkins et al, 2016), so that any high-contrast and/or colourful underwater object must be nearby.

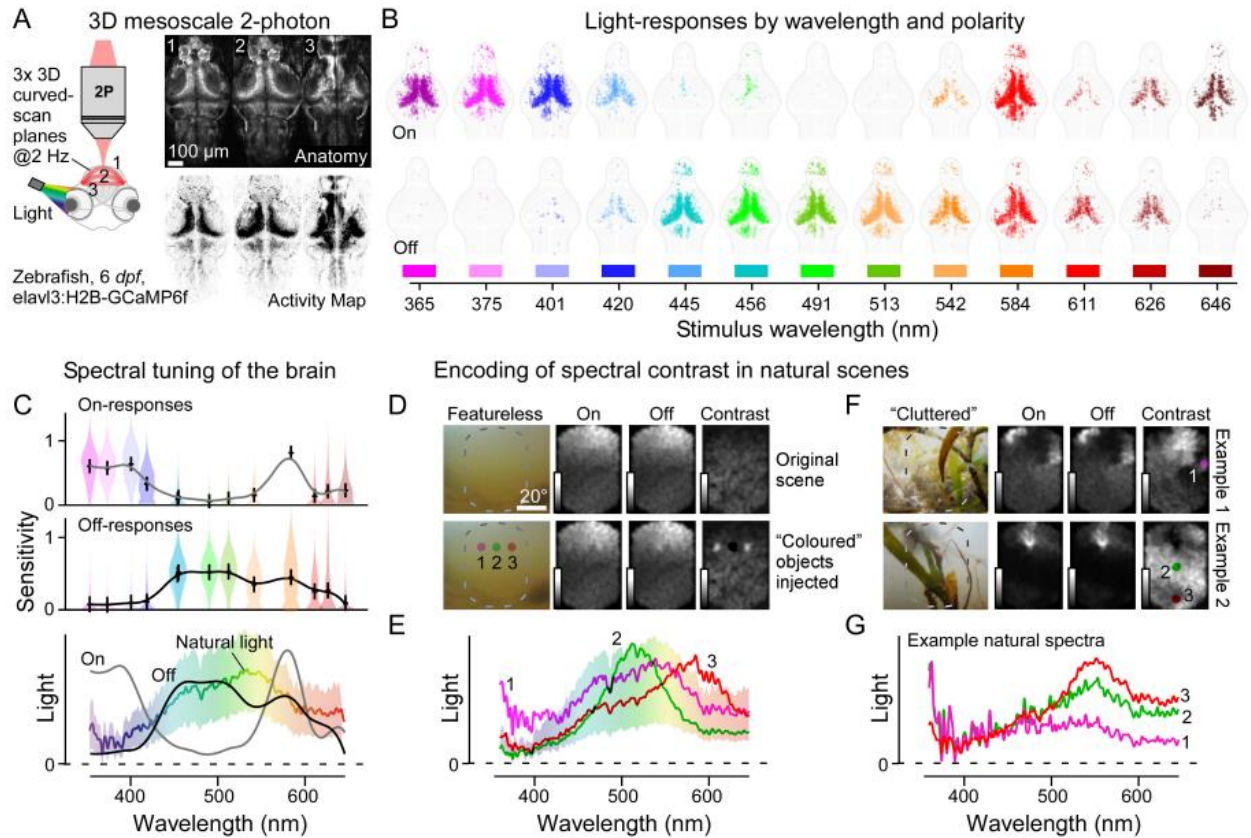


Fig 5.6: Spectral tuning of the larval zebrafish brain in the context of natural scenes.

(A) Left, larval zebrafish expressing *GCaMP6f* in neuronal somata were imaged on a custom volumetric mesoscale two-photon system with threedimensional multi-plane-bending to follow the brain's natural curvature (described in Janiak et al, 2019). Visual stimulation was by three second flashes of widefield light in 13 spectral bands (described in Yoshimatsu et al, 2020). An example brain-wide quasi-simultaneously acquired tri-plane scan average (right, top) is shown alongside a projection of pixel-wise activity-correlation (right, bottom; dark indicates higher correlation). See also Fig S5.1. (B) x-y superposition of all On- and Off-responsive ROIs (top and bottom, respectively) across $n = 90$ planes from $n = 13$ flashes of light at the indicated wavelengths. (C) Mean On- and Off-tuning functions based on (B), with crosses showing the median, and violin plots summarising the spread in the data at each wavelength (top, middle), and both tuning functions superimposed on the mean $\pm$ SD

availability of light in the zebrafish natural habitat (data from Zimmermann et al, 2018). (D–G) Selected natural visual scenes from reference8, in each case showing an indicative photograph of the scene, followed by the full hyperspectral image as seen through the On-, Off- and On-Off-contrast filters (D,F) and associated full spectra (E,G), as indicated. The bottom panels of D are identical to the top with the addition of artificially ‘injected’ local spectral distortions as indicated in E to mimic, from left to right, a ‘UV-’, ‘green-’, and ‘red-object’. Grey scalebars are 0–0.6 (black to white) for On- and Off-reconstructions, and 0–0.02 for contrast-reconstructions.

To explore this idea, we computed the mean zebrafish brain On- and Off-spectral sensitivities and compared them to the average availability of light in the zebrafish natural habitat (Zimmermann et al, 2018) (Fig 5.6C). This revealed a good match between natural spectra and the brain’s Off-filter, whereas the On-filter sensitivity peaked beyond the range of highest light availability. Nevertheless, the generally positive rectification of brain responses (Fig S5.1D,E,G) meant that both the Off- and the On-filter signals strongly correlated with brightness (Fig S5.1J,K). Accordingly, either filter in isolation encoded achromatic information, which dominates natural scenes. This correlation however also meant that when computing On-Off contrast $(\text{On}-\text{Off})/(\text{On}+\text{Off})$ as a function of wavelength, brightness information was essentially cancelled to instead highlight spectra that differed from the mean — chromatic information (Figure S5.1L).

To illustrate how such an On-Off contrast filter would serve to highlight ‘colourfulness’ in nature, we reconstructed individual natural scenes from hyperspectral images. In each case we computed three reconstructions: On-filter alone, Off-filter alone, and On-Off contrast (Fig 5.6D–G). In a featureless scene along the open water horizon, both the On- and Off-reconstructions were dominated by the vertical brightness gradient, while the On-Off reconstruction showed approximately homogeneous activation (Fig 5.6D, top). We then artificially skewed the underlying spectra of three neighbouring

regions in the same image to mimic small UV-, green- and red-biased objects, respectively, and again computed the On-, Off- and On-Off representations (Fig 5.6D, bottom, cf. Fig 5.6E). This manipulation had only minor effects on the On- or Off-reconstructions, but the contrast reconstruction readily reported the presence of all three objects. Similarly, On-Off contrast reconstructions lent themselves to reporting foliage in the foreground in non-manipulated, cluttered natural visual environments (Fig 5.6F,G).

Taken together, our data suggest that the zebrafish brain's overall spectral On-Off tuning is suited to represent the presence of spectral information that differs from the mean, and thus to provide a cue to object 'colourfulness', which in turn correlates with object proximity (Wilkins et al, 2016). Beyond this overarching spectral response profile, substantial additional spectral diversity exists at the cellular and neurite level, presumably to support the zebrafish's various visual requirements (Zhou et al, 2020. Guggiana et al, 2021, Fornetto et al, 2020).

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Declaration of interests

The authors declare no competing interests.

Supplemental Information

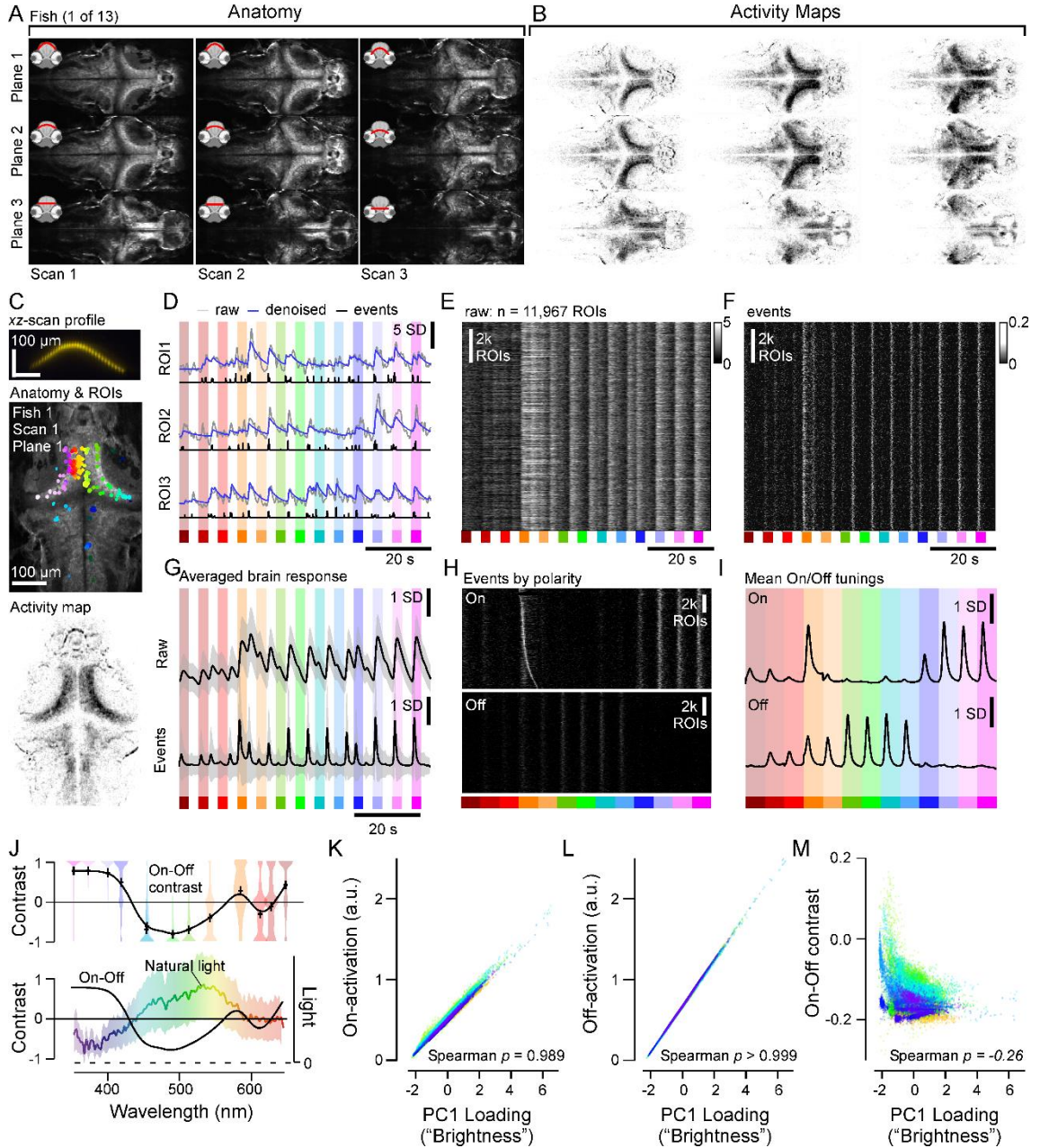


Fig S5.1: Measuring the bulk spectral tuning of the larval zebrafish brain.

A, Example recordings from one larval zebrafish, comprising three consecutive scans of three planes each for a total of nine planes. For each triplane scan, starting from a common z-position, the first two planes were bent upwards by $\sim 100 \mu\text{m}$ and $\sim 50 \mu\text{m}$ at the apex, respectively. The lowermost plane was kept flat. Between scans, the entire triplane was moved down by $\sim 50 \mu\text{m}$. In total, we recorded from $n = 13$ fish in such a configuration. **B**, Pixel-wise activity-correlation over time with the four neighbours

computed as in Franke et al (2017) as an indication of locally correlated activity in the scan (darker shade indicate higher correlation). **C**, Example ROI extraction shown for plane/scan/fish 1 (cf. A,B), with xz-scan-profile visualised as in Janiak et al (2019) (top), a crop of the anatomical projection with ROIs (middle) and corresponding activity map (bottom). **D**, Example ROIs from (C) in response to light-flashes of different wavelength as indicated, shown as z-normalised fluorescence (grey), denoised (blue) and detected events (black). **E,F**, All $n = 11,967$ ROIs from 13 fish (30 scans) shown as raw fluorescence (E) and as events (F). Note polarity switches between light-flashes of different wavelengths. **G**, Mean $\pm$ 1SD z-normalised fluorescence (top) and events (bottom) of all ROIs. **H,I**, Separate On- (top) and Off-event phases (bottom) extracted from (F,G) as heatmap (H) and mean tuning (I). All ROIs are sorted by the timing of the On-event in response to 584 nm (“peak” orange/red) light stimulation. In all heatmaps showing ROIs, lighter colours indicate a higher signal. **J**, as Fig 1C, but showing On-Off contrast (i.e. the “brain filter”). **K-M**, Activation of the On- (J), Off- (K) and On-Off Contrast-filters (L) for each of 30,000 individual natural spectra (from $n = 30$ scenes^{S3}) plotted against their “brightness”, here computed as their loading against the first principal component (PC) that emerges from PCA across the entire dataset (see also Ref^{S3}). Data from individual scenes is indicated by their different coloration. Spearman correlation coefficients ρ as indicated.

Supplemental Experimental Procedures

Resource Availability

Lead Contact. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tom Baden (t.baden@sussex.ac.uk).

Data and Code Availability. Pre-processed functional 2-photon imaging data and associated summary statistics are freely available at <https://datadryad.org/stash/dataset/doi:10.5061/dryad.4xgxd2584> and via the relevant links on <http://www.badenlab.org/resources> and <http://www.retinal-functomics.net>. The natural imaging dataset was published previously as part of Zimmermann et al (2018).

Experimental Model and Subject Details

Animals. All procedures were performed in accordance with the UK Animals (Scientific Procedures) act 1986 and approved by the animal welfare committee of the University of Sussex. For all experiments, we used 6-7 *days post fertilization (dpf)* zebrafish (*Danio rerio*) larvae. The following previously published transgenic line was used: Tg(elavl3:H2B-GCaMP6f); ZFIN ZDB-ALT-150916-4^{S4}. Animals were housed under a standard 14:10 day/night rhythm and fed three times a day. For 2-photon *in-vivo* imaging, zebrafish larvae were immobilised in 2% low melting point agarose (Fisher Scientific, BP1360-100), placed on a glass coverslip and submerged in fish water.

Light Stimulation. With fish mounted upright, light stimulation was delivered as wide-field flashes from a spectrally broad liquid waveguide with a low NA (0.59, 77555 Newport), positioned next to the objective at $\sim 45^\circ$. The other end of the waveguide collected light from 13 “spectrally narrowed” LEDs, as described in detail elsewhere (Yoshimatsu et al, 2020). All stimuli were series of single LED flashes of light lasting 3 s, separated by gaps of 3 s (1 stimulus loop: 13 LEDs * (3+3) s = 78 s. 3-4 loops were presented and averaged for each recording.

2-photon calcium imaging. All 2-photon imaging was performed on a MOM-type 2-photon microscope (designed by W. Denk, MPI, Martinsried; purchased through Sutter Instruments/Science Products) equipped with a mode-locked Ti:Sapphire laser (Chameleon Vision-S, Coherent) tuned to 960 nm for SyGCaMP imaging. We used one

fluorescence detection channel (F48x573, 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 6.3 for Windows (Wavemetrics).

To expand the field of view to ~ 1.2 mm diameter, which allowed capturing the entire brain's length in a single scan, we used a non-telecentric optical approach as described in detail elsewhere (Janiak et al, 2019). The excitation spot (point spread function) in this configuration was ~ 0.7 μm (xy) and ~ 11 μm (z) at full width half maximum. This optical configuration can in principle capture the signals from individual larval zebrafish somata^{S2}. However, in this work it was our intention to capture the bulk spectral responses across large fractions of the brain. Accordingly, we balanced recording area and spatial sampling such that individual somata effectively corresponded to single, or at most groups of 2-4 pixels (3 planes covering $\sim 450 \times 1,000$ μm with a 160×350 px scan each to yield ~ 2.9 μm voxel xy-spacing, compared to average zebrafish neuronal soma diameter of ~ 7 μm ; 1 ms per line, 2.08 Hz volume rate).

To follow the brain's natural 3D curvature, we also systematically 3D-bent each scan-plane as a function of the slow scanning-mirror's position to form a "half-pipe". Curvature was achieved via rapid remote focussing synchronised with the scan pattern, as described in detail elsewhere (Janiak et al, 2019). The degree of peak axial curvature was empirically adjusted between 0-150 μm between scans and planes to achieve best overall sampling of the entire brain.

Pre-processing and extraction of response amplitudes of 2-photon data.

Recordings were linearly interpolated to 42 Hz and manually aligned between fish using a time-averaged brightness projection. Regions of interest (ROIs), corresponding to individual and/or small groups of neighbouring neuronal somata were defined automatically using custom Python scripts. In short, we used a "quality-index" (QI,

described in detail in Baden et al, 2016) to first identify individual pixels that exhibited reliable responses to repeated stimulation. For this, we computed a pixel-wise QI-projection of the deinterleaved recording, sorting QI-pixels in descending order. The resulting curve was differentiated using *scipy.interpolate.splrep*. Pixel indices between inflections of the differential were projected back into space. Contours were identified using dilation (3,3)-erosion(2,2) and contour finding of Python-OpenCV. Individual contours were taken as ROIs, discarding any ROIs with a diameter > 15 μm . QI per ROI was then recalculated and used for further thresholding at $\text{QI} > 0.5$. From here, fluorescence traces were extracted and z-normalized based on the 6 s at the beginning of recording prior to stimulus presentation. Overall, this strategy served to balance the need to combine multiple pixels into ROIs to boost their signal-to-noise, with a goal of keeping ROIs as small and localised as possible to approximately report the signals single, or from at most very small groups of somata that responded in a similar manner. This compromise was necessary to accommodate the large size of the scan pattern capturing the entire length of the brain while also maintaining a reasonable imaging rate. A stimulus time marker embedded in the recording data served to align the traces relative to the visual stimulus with a temporal precision of 1 ms.

Separation of On- and Off responses. Calcium traces were deconvolved using ARMA(1) (*caiman.source_extraction.cnmf.deconvolution*, Giovanucci et al, 2019). Inferred discrete events were partitioned into events occurring during stimulus presentation and the complement.

Computing the brain's bulk spectral tuning functions. Inferred events were summed over respective stimulus time windows. Sums were averaged over all recorded traces. Contrast between On and Off portions of the response was calculated as their difference over their sum.

Natural Imaging Data Analysis. Hyperspectral data were obtained from Ref^{S3} and element-wise multiplied with a deuterium light source derived correction curve (see online data). The data were restricted to the domain of 360-650 nm. Here, the long-wavelength end of the domain was decided based on the long-wavelength opsin absorption curve; the short-wavelength end was dictated by the sensitivity of the spectrometer. Spectra were scaled by standard deviation within a given scene. Traces were multiplied with the respective On- and Off-filters. The responses were summed within spectrum to produce a single number per point spectrum (or 800-long vector per scan). These vectors were standard-deviation-scaled within a scene. Spatial projections of filter responses were Gaussian-smoothed in space ($\sigma=2\text{px}$).

6. Conclusions

Summary

We have systematically investigated the visual pathway of the zebrafish with regard to its spectral response characteristics. We have uncovered that:

- *In vivo* pedicles efficiently represent chromatic information via rotation of the response axis away from the opsin response axis.
- UV light representation is ubiquitous and 'special'.
- Bipolar Cells signal diverse chromatic information. There is clearly greater regional anisotropy in BC responses than there is in cone responses.
- Most of this response diversity is lost in the brain, conditional to the stimulus presented.
- Previous ideas about functional anatomy of the zebrafish: On-Off layers of the IPL and the "Optic Tectum" - should be revised to include wavelength as a factor. On-Off layering is subject to stimulus conditions and so is tectal lamination.
- The spectral sensitivity profile of the zebrafish is intrinsically opponent under full-field stimulation.

In other words, the quest that we took upon ourselves, namely, high spectral resolution investigation of the zebrafish visual system, has proven to be a fruitful one.

Information Efficiency and Teleology

We explain the functional cone data via information efficiency with respect to sampled points in the visual world. We expressed downstream brain neuron functional data explanation in teleological terms. The two accounts are not incompatible. Assuming that every neuronal layer is optimal with response to some stimulus feature AND that this feature is represented in the presented stimulus AND that responses of different layers are not identical; we arrive at the conclusion: different neuronal layers are

optimal with respect to different stimulus features. In other words, different explanations should be provided for different layers.

Let us say that cones maximise information transmission with respect to separate pixels in the image. We expect filters sensitive to particular visual features to be combinations of the classical achromatic and chromatic visual channels. Feature coding by extension maximises information transmission with respect to particular features or objects in the image.

Objects are defined through their properties and in no other way (e.g., Just Noticeable Difference, “1.1 The world is the totality of facts not of things.” Wittgenstein, *Tractatus Logico-Philosophicus*). The properties accessible to a zebrafish larva are limited by the physiology of the zebrafish eye: its effective visual acuity, dictated by photoreceptor spacings, is on the order of 2-3 degrees of visual angle (Haug et al, 2010). Feature coding is dependent on the accessibility of real-world features to the fish. One naturally expects to find primarily achromatic bar and edge detector neurons - but that is not guaranteed. As Maximov (2000) argues (see Ch1), differently tuned chromatic sensitivity provides increased object edge affordability. Generally, the receptive field properties that reflect “What does the eye see best” (Watson et al, 1983) can look unexpected to the human eye; because what is defined as an object depends on the species and the maturity of the individual animal. As examples: human infants do not gain object permanence until several months of age (Baillargeon et al, 1985); neural networks that are taught to recognise objects often achieve it via very unexpected receptive field structures.

What is the overall algorithm governing transformations of spectral information in the zebrafish?

We argue that PR pedicles achieve PCA-like rotation of the spectral space. Other ideas exist. Following Dinemaller (1992) one can argue that PR pedicles achieve colour

constancy. Indeed, this has been proposed (Howlett et al, 2016). I think that the PCA-hypothesis is the more conservative one at least in the larva, and is thus preferable.

The following should be considered:

1. Dannemiller(1992) shows that colour constancy under different illuminants can be easily achieved because most of the change in the natural illuminant bears on a factor equivalent to our “natural image” PC1 (Ch.3). This systematically does not hold for the zebrafish. The proportion of variance in PC2 for the zebrafish visual world is significantly larger than the one for humans. This is in part due to the fact that loading onto PC2 systematically varies with depth for the shallow water zebrafish (Zimmermann et al, 2018). Perfect colour constancy would require an appropriate PR adaptation. It is unknown whether the G-cone peak variation in the zebrafish retina accommodates this requirement. In addition, colour constancy was modelled for underwater conditions, and it was found that von Kries transformation fails to adapt to underwater distance-dependent colour changes (e.g. Vorobyev et al, 2001, Wilkins et al, 2016).

2. Typically measures of the illuminant do not include the UV band, due to measurement difficulties. Our measurements suggest considerable signal power in this band. Indeed, it seems to have at least one latent factor in common with our PC1 (Fig 5.6, S5.1). If this is indeed so, under colour-constancy account one could expect strong opposing UV-inputs to at least some R-G contrast neurons (to account for the illuminant), which is not observed *in vivo*. Indeed, for colour constancy with four cones we expect to find a greater number of chromatically-opponent profiles. While the proposed PCA hypothesis can not readily explain the presence of the UV-cone, neither can the colour-constancy hypothesis.

3. Wilkins & Osorio (2019) argue that matter-identity is the deciding factor for the chromatic sensor structure. Indeed, their measure will definitionally require

psychophysical contrast between UV and other spectral cones. This, however, need not strictly hold *in vivo* as observed. Information efficiency simply requires that most information is transferred through as few channels as possible. That is well in line with our findings. A contrast required for a specific detector can be computed downstream, given that the UV information is still available. Indeed, this is what we find *in vivo*. Identity or affordances of UV-variable portions of the visual field are, seemingly, computed ubiquitously in the brain (Ch.5).

For all these reasons I believe that we have achieved a good and conservative first explanation.

The question remains, nevertheless, of why chromatic rotation ‘takes place’ before the spatial one. I.e. should one take PCA on a 3D image (width, height, colour), the first components will be largely achromatic. The number of such achromatic components is a function of the size of the image (scaling with the maximal frequency where substantial signal power is to be found). If one breaks down the image into small enough patches one will recover components that look like edge- and chromaticity-filters. That at first, image is decomposed into patches and not processed somehow globally seems to be a fact of neuronal wiring. I.e. first neurons in the visual pathway process the image locally and not globally.

In other words, there is an answer to this question: ‘The neuronal wiring is this way’. This answer is about the implementation level (in D. Marr’s terms). The question of the overarching algorithm is not answered, to the best of my knowledge.

Temporal richness of chromatic pathways

As we argue in Ch.4 little chromatic transformation takes place in BCs, most of the variation comes from differences in the temporal response profiles of the BCs. I will speculate what significance it has.

Most of the temporal response profiles systematically vary with wavelength (Ch.4, Fig 10). Why would it be good for a cell to have responses that vary in phase?

Consider some cell A that is targeted by Red and UV signals. Let's say these signals are in-phase. The source of the incoming signal is only represented in anatomical connections and the response amplitude of the cell A (let's assume that the two sources sum together perfectly in the cell). Hence, a kind of 'univariance' holds within the cell. Such a system corresponds to a logical $\vee$, inclusive disjunction.

Now consider that the signals are out-of-phase. The source of the signal is temporally encoded. Sustained signal corresponds to a logical conjunction, $\wedge$. As discussed before in the Introduction, the latter allows for representation of complex spectra within-cell and the former does not.

Interestingly, such temporal asynchrony is used to learn predicates in biologically-inspired settling networks (Doumas et al, 2008). Suppose that at the level of GCs spectra are represented in normal logical forms (NLF) (Disjunctive and Conjunctive Normal Forms) for ease of computation in progressively larger RFs. This is sensible because NLF allows for certain efficient computations (for a good reference on the topic, consider Stoll, 1979). Such computations naturally allow for complex RFs. Then one would predict that GC coding will be the logical dual of BC coding (i.e. logical conjunction of disjunctions and vice-versa). Hence, the temporal richness of BCs should not come as a surprise. Of course, I present this simplified thought experiment as mere speculation.

Sensitivity and the grey point

It should be noted: one expects to find a grey-point specification in a colour-vision investigation. Instead, the stimulus used throughout this investigation uses darkness as background. Such procedures have been carried out primarily with PRs as preparation. So, in what sense are we talking about colour opponency? First, experimenters were

able to distinguish and name colours under presentation conditions. There is nothing in the conditions that precludes a colour-opponent system to distinguish between different colours.

One has to consider PR sensitivity to address this question properly: the UV cone is clearly the most sensitive one. This is well in line with previous data (e.g. Nelson & Singla, 2001). Does UV then adapt the zebrafish visual system more than any other wavelength band? We do not know what adapted state of the larval zebrafish visual system the grey point maps onto yet.

It is not unreasonable to suggest high-gain postreceptoral mechanisms to explain this increased sensitivity in laboratory conditions (consider Howlett et al, 2016). I will speculate a different mechanism. There is evidence of a wide-reaching syncytium-like structure directly between UV-cones in the zebrafish (T.Yoshimatsu, personal communication). Could, instead, the UV signal be boosted through increased spatial summation in the UV cones? Considering the amount of visual scatter in the UV band, it is not unreasonable to suggest so. This would mean that UV cone RFs are larger than other cone RFs. Temporal frequency of visual filters is predicted to be decreased from achromatic to chromatic ones (Atick, 1992). Same goes for spatial filters for reasons of increasing Signal-to-Noise Ratio.

In conclusion, additional experiments need to be carried out both behaviourally and physiologically to construct a definition of grey point that will account for all these possibilities.

Afterword

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