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Visual Behavior in Zebrafish

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ABSTRACT

The zebrafish mainly uses the sense of light to hunt for food and avoid predators. This reliance on vision necessitates the rapid development of the visual system. Therefore the early larva already exhibits a number of visually-mediated behaviors that can be used for a genetic analysis of vision. The present is an overview of the properties of the zebrafish visual system in the context of its use for behavioral based screens.

INTRODUCTION

THE ZEBRAFISH ENJOYS THE STATUS of one of the most widely used vertebrate model systems in developmental biology. The ease of forward genetics in combination with the rapid development of the transparent embryos is the basis of its appeal to developmental biologists. In addition, in the last few years reverse genetic tools have been established to complement these genetic approaches. More recently, the interest in zebrafish behavior is growing (exemplified by this special issue of ZEBRAFISH), mainly due to the prospect of using the zebrafish model for behavioral genetics. Such an approach was pioneered more than 30 years ago in invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*.[1,2]

Among the readily accessible behaviors that can be approached by genetics are visually-mediated behaviors. Light, the relevant external stimulus for the visual system, can be easily controlled in stimulus quality (wavelength of light, perceived as color) and intensity (irradiance). The eye as the main organ of light perception is an accessible part of the central nervous system and well defined in its cellular composition and morphological arrangement.[3] Furthermore, vertebrates display a number of well-defined behavioral responses of varying complexity to visual stimulation.

The zebrafish visual system develops extraordinarily rapidly, resulting in a well developed and functional visual system of the 5-day-old larva. This developmental stage coincides with the depletion of the yolk supply. The precocious development of the zebrafish visual system can be seen as an adaptation to the ecological pressure of the young free-swimming larva to hunt for food, since prey capture is thought to be mediated by vision at larval stages.[4]

The following section is an overview of the properties of the zebrafish visual system including its development. This sets the stage for a review of visual mediated behaviors with an emphasis on how such behaviors can be used for a genetic analysis of vision.

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DEVELOPMENT AND ANATOMY OF THE ZEBRAFISH VISUAL SYSTEM

The size dominance of the eyes relative to other body structures of the larval zebrafish already hints at the importance of the visual system for the larva. The larval zebrafish retina at 5 days postfertilization (dpf) shows the typical layering and contains all the cell types of vertebrate retinas (Fig. 1). The outer retina contains the photoreceptors, which can be grouped into rod and cone photoreceptors. While there is only one anatomical rod type using rhodopsin as its chromophore, cones can be anatomically subdivided into four different types: short single (SSC), long-single (LSC) and the two members of the double (DC) cones. These cone types are also characterized by their visual pigment content, with peak sensitivities in the ultraviolet, short-, middle-, and long wavelength (UV, S, M, L), respectively. The peak sensitivities have been measured with slightly variable values for both rods (501–503 nm) and cones (UV cone, 360–361 nm; S cone, 407–417 nm; M cone 473–480 nm; L cone, 556–564 nm).[5–8]

Hence zebrafish vision is tetrachromatic, while human trichromatic vision lacks sensitivity to ultraviolet light.

Interestingly, although rod photoreceptors are morphologically detectable in the 5 dpf retina, they appear to contribute little or nothing to vision.[9,10] Rod contributions are only measured at stages older than 15 dpf.[9] Therefore the larval zebrafish retina can be seen as a functional cone-dominant retina. This opens the possibility to specifically analyze cone-mediated vision, which is more difficult to study in the rod dominant nocturnal mouse.

In the second cell layer, the inner nuclear layer, the cell bodies of bipolar, horizontal, and amacrine interneurons are found, as well as the cell soma of Muller glia cells, the main glial population of the retina. Synaptic contacts between photoreceptors and the inner retina are formed in the outer plexiform layer. The cell layer closest to the lens is the ganglion cell layer, containing displaced amacrine cells and ganglion cells that form long axons comprising the optic nerve and (outside of the retina) the optic tract. Synaptic contacts between ganglion cells and the inner nuclear cells are formed in the inner plexiform layer and its thickness hints at the great complexity of these diverse synaptic connections.

The initial event of eye development is the evagination of the optic lobes from the diencephalon at around 10 hpf (hours postfertilization). Thereupon ganglion cells of the ganglion cell layer differentiate at about 32 hpf[11–14] and their axons reach the optic tectum shortly thereafter. At 50 hpf the first amacrine and horizontal cells of the inner nuclear layer appear, and by 55 hpf the appearance of rod and cone outer segments in the outer nuclear layer as well as rod and cone synaptic terminals can be detected.[14] Functional ribbon triads arise within photoreceptor synaptic terminals at 65 hpf and finally, bipolar cell ribbon synapses form at about 70 hpf. Signal transmission from photoreceptors to second order neurons starts around 3.5 dpf and is fully functional at 5 dpf.[15]

Embryonic visual system development can be described in anatomical terms of neuronal and synapse formation. All these developmental steps are also reflected in the step-wise occurrence of visually-guided behaviors.

The emergence of functional ribbon synapses marks the time point at which the first visually evoked behavioral response, the startle response, can be elicited. In their startle response, larvae respond to a sudden increase of illumination by rapidly turning away from the light source.[16]

Coinciding with a differentiated and synaptically layered retina, first optokinetic responses

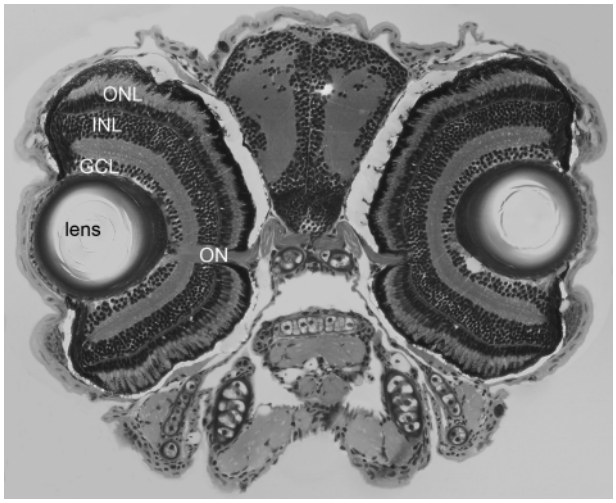

FIG. 1. Morphology of the visual system of a 6-day-old zebrafish larva. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve.

(OKR) can be measured at 3 dpf.[17,18] Optokinetic responses are tracking eye movements evoked by motion in the surround. Also at this time, the first sum field potential of the eye in response to light (electroretinogram, ERG) can be recorded.[10] However, full fledged ERGs can only be measured at slightly later stages,[19] coinciding with functional signal transmission between photoreceptors and second order neurons.

Larva also show a tendency to swim in the direction of moving stripes, a behavior termed the optomotor response.[20–22] This behavior is robustly displayed only at stages older than 6 dpf, suggesting that the responsible neuronal circuits (possibly located outside of the retina) develop slightly later than the ones mediating the OKR.

The adult zebrafish retina contains no additional cell types that are not present at larval stages. The pattern of the adult zebrafish outer retina resembles a mosaic of cones interspersed with rods.[23–25] An important difference to the larval retina is the contribution of rods to visual function. Initial rod contribution can be measured first at 15 dpf, but they do not become adult-like until around 30 dpf.[9,10]

IMPACT OF EXTRINSIC AND INTRINSIC FACTORS ON ZEBRAFISH VISION

Effect of light on visual system development

Exposure to constant light or constant dark rearing-conditions during embryonic development leads to deficits in visual behavior,[26] as well as to reduced spectral sensitivity in zebrafish larvae.[27] Fish that are reared in constant light suffer from great deficits in UV and S cones, whereas slight deficits across the entire spectrum are obtained in fish reared in constant dark conditions. Amazingly, zebrafish are capable of regenerating their spectral sensitivity after few days.

A recent study by Dixon et al.[28] confirmed the detrimental effect of constant light rearing on UV and short wavelength sensitivity. Determining the impact of cyclic light on the larval visual system, they were able to show that cyclic green or orange light conditions induce strong deficits in the sensitivity of the UV and short-wavelength spectrum, whereas cyclic blue light has no effect. Thus, cyclic short-wavelength light is required for proper visual system development.

Circadian clock

There is strong evidence that a variety of different mechanisms of the zebrafish visual system are regulated by an endogenous circadian clock. Examples are melatonin synthesis, which is elevated at night and downregulated during the day,[29] or increased expression of IRBP (interphotoreceptor retinoid binding protein) mRNA during the day compared to the night.[30]

Clues about the influence of the circadian clock on visual system performance were also observed in studies of visual behavior and ERG sensitivities. The threshold light intensity for detection of visual stimuli as assayed by the adult escape response is higher during night compared to day, which argues for elevated sensitivity to light onset during daytime.[31] Hence, the time of the day has to be considered for any adult visual performance assessment. This circadian influence is also mirrored by ERG studies on b-, d- wave dominance transition. The b-wave mainly reflects the ON response at light onset, while the d-wave mainly reflects the OFF response at the cessation of light. In the course of dark adaptation following bright light adaptation, dominance of ERG b- and d-wave switches.[32] Whereas both waves appear similar in size in early dark adaptation, d-wave amplitude decreases in size in late dark adaptation. The time course for this rhythm varies between day and night, being rapid in late afternoon and slow at night and early morning. The described visual behaviors as well as ERG b/d wave transition persist when fish are kept in constant light, arguing for an endogenous circadian clock, working to decrease visual sensitivity to light onset during night.

BEHAVIORAL GENETIC APPROACHES TO ZEBRAFISH VISION

George Streisinger, who had pioneered the use of the zebrafish as a model system in the 1970s, already realized the great potential of the zebrafish for visual behavioral screens. He and his

colleagues were the first to employ behavioral assays like the optokinetic and optomotor response to uncover potential vision mutants.[33] Subsequent behavioral assays have been used in several forward genetic large-scale screens.[21,34,35]

Nowadays, a zebrafish scientist can choose from a variety of established and robust behavioral tools to screen for mutant strains with defects in vision. Likewise, by using the same assays in more specific ways, fine-tuned characterization of mutants by varying stimulation conditions can be achieved and genetic defects can be located specifically in the visual pathway. For instance, specific aspects of vision, such as light adaptive properties, contrast sensitivity, and visual acuity can be measured by more detailed behavioral analyses.

Given that behavioral assays are noninvasive, the larva can be subjected to an electrophysiological (ERG, patch-clamp) or histological analysis following the behavioral assessment of the very same animal.[36]

The following section will give an overview about the variety of behavioral assays that are used to date.

General considerations

In order for a behavioral assay to qualify for a genetic screen, it has to meet a number of requirements. The behavioral response should be ideally robust (elicited any time the stimulus is given), fast to assess, and as specific as possible for the sensory property of interest. Reflexive behaviors, such as the OKR, tend to be more robust, but likely probe simpler neural circuits. The statistical nature of Mendelian genetics necessitates the screening of a large number of animals per family. Hence, population assays enabling the screening of many larvae in parallel are preferable. However population assays only work if individual animals of the group behave independently from each other. Since adult zebrafish display both schooling and dominance behavior, population assays are only possible in larvae.

Visual information is deconstructed and processed in parallel channels in the vertebrate visual system.[37] This segregation allows for the separate testing of visual processing channels. However, so far mainly motion stimuli have been used to probe the zebrafish visual system. In order to more directly address aspects of visual processing in the brain, more refined behavioral paradigms are needed. Such paradigms will likely be more complex and the resulting behavioral responses more ambiguous, thereby exacerbating such forward genetic screens.

One way to partially alleviate this problem is to devise a two level screening strategy by defining exclusion criteria. For instance, a recent extensive screen for visual behavioral defects excluded all animals with visible morphological abnormalities for the behavioral analysis.[38] Similarly a population assay can be used to preselect strains for a more specific or in-depth analysis.

Visual background adaptation

Morphological inspection of zebrafish larvae can give a first hint to potential blindness. This is due to the ability of larvae to adapt to their respective background.[39] Melanophores in the skin of zebrafish adjust the distribution of their melanosomes according to the background, mediated by a hormonal response. On a dark background, melanosomes are widely distributed and thus the larva appears darker than on a more light background, when melanosomes are contracted. Blind zebrafish cannot sense the level of background light and thus a great percentage of blind mutants appear black. However, this criterium is not sufficient, since a number of hypopigmented and normally pigmented blind zebrafish mutants have also been identified.[40,41] Since background adaptation depends simply on sensing light, irrespective of other qualities (motion, form, color), it cannot address questions of more complex visual processing (e.g., contrast detection).

Optokinetic response (OKR)

A very robust assay of visual system function is the optokinetic response (OKR). Starting at about 3 dpf, zebrafish larvae track movements in their environment with their eyes. Due to the large pigmented eyes, these eye movements can be easily followed under a dissecting scope. This behavior has been used in a number of screens, typically by placing the

larvae inside a rotating drum fitted with black and white stripes.[21,35,38] The larva is immobilized in a viscous solution (typically methycellulose) in order to prevent it from swimming. The rotating drum elicits a stereotyped behavior, the optokinetic nystagmus (OKN), a subtype of optokinetic responses. This stereotyped behavior consists of two separate eye movements: a smooth pursuit movement in the direction of the rotation and a fast reset movement after the image left the visual field.

Such screens have led to the isolation of a number of visually impaired mutant strains (reviewed in Refs. 20 and 42). This assay can be adapted to screen for mutants defective in color vision. Such a screen, using gratings illuminated with long-wavelength (red) instead of white light, led to the isolation of a red-light insensitive mutant.[43] This mutant (*partial optokinetic nystagmus* (*poa*)) did respond to white light but not red light. Such an approach is limited to wavelengths of light that elicit responses of a specific cone type. Hence, it can only be applied to wavelengths at the extreme of the visual spectrum (red light), where roughly a single cone type is responsive.

For the initial screening of larvae, a qualitative assay is sufficient. However, a more detailed analysis calls for a quantitative assay, particularly for mutants with problems in visual processing rather than light sensing. The OKR can be adapted to measure visual performance quantitatively.[44,45]

For this purpose, the rotating drum is replaced by stripes projected onto a screen viewed by the larvae (Fig. 2). Real time semi-automatic measurements of eye velocity can then be correlated to visual stimulus properties and thereby measured quantitatively. The gain of this behavior, defined as the ratio between stimulus and eye velocity, serves as a quantitative measure of visual performance. Such an analysis revealed that the gain of the OKR slow phase depends on angular stimulus velocity, spatial frequency (a measure of stripe width), and contrast of the moving grating. By establishing these functional relationships, such measurements can be used to quantify visual

F2

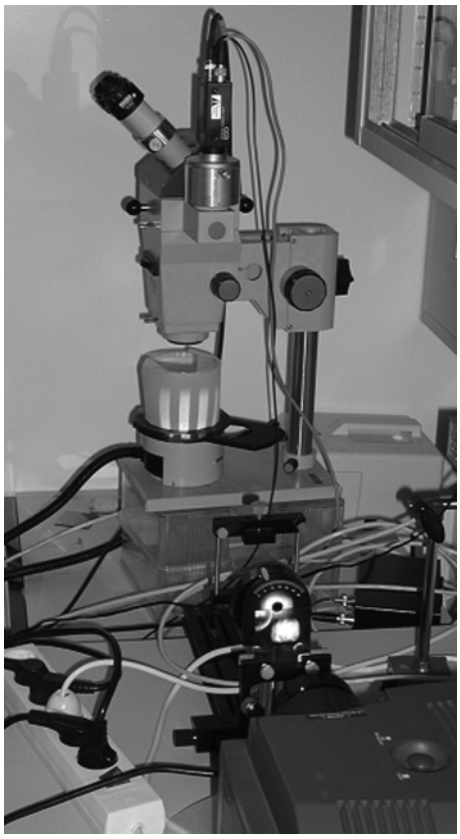

FIG. 2. Larval optokinetic response (OKR). The immobilized larva faces a moving black and white grating, which is projected onto the circular tank by a digital light projector (a commercial video beamer). Light intensity can be varied by inserting neutral density filters into the optic path. Eye movements are recorded online with a CCD camera.

performance such as visual acuity, contrast sensitivity, and light adaptation.[45] Hence, the OKR can be used for probing special features of visual performance and becomes a valuable tool for a detailed analysis of visual system defects in mutant and morphant larvae.

The OKR is ideally suited for the analysis of zebrafish larvae but is not readily applicable for adults. The oxygen supply of the larva before scales are formed is easily met by oxygen diffusion through the skin. Hence, larvae can be embedded in methylcellulose solution without any obvious signs of distress. Even embedding for a prolonged time (24 h) does not affect survival. This situation is different for adults. Here, oxygen needs to be supplied through the gills and hence any embedding that would plaster up the gills will harm the fish. Any method to measure a robust adult OKR will have to establish a behavior compatible way to restraining the fish. Such methods have been applied to other fish but not to zebrafish yet (e.g., in goldfish[46]).

Larval optomotor response (OMR)

The OMR (optomotor response) is a well-established and useful tool to probe zebrafish larvae for blindness. In order to elicit an OMR response in zebrafish larvae, movies of directionally moving alternating white and black stripes are presented to the larvae either from below or from the side.[21,47] The majority of 7 dpf wild-type larvae will swim in the direction of the perceived motion, either following a leading or escaping a trailing stripe. Hence, nonresponsiveness to the moving grating implies larval blindness. Several families can be tested simultaneously. The population response can be evaluated by assessing the distribution of the larvae. Larvae with intact vision will tend to cluster at one side of the lanes, determined by the apparent direction of the moving grating (Fig. 3). These features make this assay well suited for large-scale screening approaches, since a number of clutches can be screened in parallel.

F3

In addition, the OMR can be used for fine-tuned analysis of the zebrafish larval visual system. Since the stimulus is computer generated, the properties of the moving grating can be varied conveniently. Therefore, contrast and wavelength dependent properties of the OMR can be readily investigated.[22] A robust OMR can only be evoked as late as by 7dpf and thus is less suitable for screens aiming at identifying mutations that affect early larval survival.

Adult OMR

The OMR can also be applied to adult zebrafish. However, schooling makes population screening impractible. Single adult zebrafish are placed inside a round testing chamber surrounded by a rotating drum containing alternating black and white stripes (Fig. 4). Fish have the tendency to swim in the direction of the stripes, which can be used as a measure of visual performance. For example, Maaswinkel and Li used this assay to establish the relationship between spatial and temporal frequencies in motion detection.[48]

F4

Another report applied this behavioral paradigm to investigate the contribution of color to motion detection.[49] The authors concluded from their data, that chromatic inputs to the OMR in adults are exclusively fed by red cones, similar to findings in goldfish.[50] This situation is different in larvae, where both red and green cone input feed into the OMR[47] (and see below). This could hint at an interesting difference in visual processing between larval and adult stages.

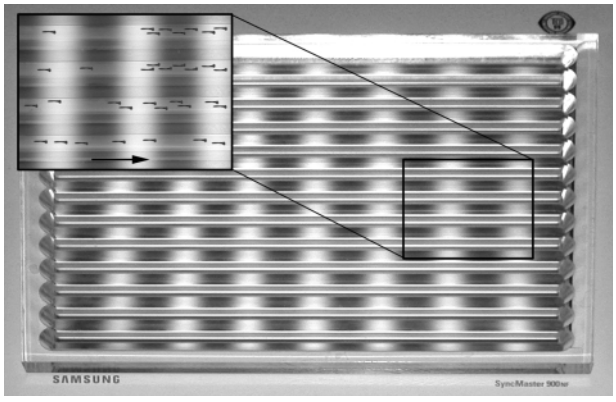

FIG. 3. Larval optomotor response (OMR). The plexiglass swimming arena is placed on a tipped computer monitor. Displaying moving stripes on the screen elicits swimming of the larvae in the apparent direction of motion, so that larvae are concentrated on one side of the lanes (*inset*).

FIG. 4. Adult optomotor response (OMR). A single adult zebrafish is placed into a circular tank surrounded by a moving drum fitted with black and white stripes. The fish will swim with the moving stripes.

Adult escape response

Zebrafish initiate an escape response when threatened by a potential predator. This innate behavior can be used to assess visual performance. This behavioral assay was established for screening purposes by Li and Dowling.[51,52] In an experimental set-up, the zebrafish is placed into a round testing chamber and the threatening object is mimicked by a single black stripe on an otherwise white circular rotating drum (Fig. 5). By varying the illuminating light intensity, absolute rod and cone system thresholds as well as the course of dark adaptation after bright light adaptation can be measured.[51] A screen of chemically mutagenized F1 fish for dominant mutations altering visual sensitivity yielded a set of mutations with progressive dominant retinal degeneration.[51–54] Such mutations are of high medical relevance, since most human outer retinal dystrophies are progressive and manifested in older patients.

F5

COMPARATIVE ASPECTS OF ZEBRAFISH VISION

The morphology of the visual system, in particular the retina, is very well conserved throughout vertebrate species. However, at closer inspection, the visual system of vertebrates is surprisingly diverse in functional properties (e.g., by the absorption characteristics of its photoreceptors[55]). This likely reflects the high selective pressure imposed on the visual system, resulting in a fine tuned matching of visual system properties to the respective ecological niche of the animal. A comparative approach to understand the evolution of vision is therefore promising. Although the study of visual processing in higher vertebrates is a very active research area, comparatively little is known about it in lower vertebrates. The question of how comparable lower and higher vertebrate vision really is, lies at the heart of any proposal to use the zebrafish as a human disease model. Recently, some of these questions have been addressed by behavioral experiments in zebrafish.

Motion perception

Motion detection is dominated by first-order (luminance-defined or Fourier) motion cues, but there is much evidence that humans can perceive complex movements in the environment by using second-order features, such as movement of contrast, texture, or flicker.[56] This phenomenon of second-order motion,[57] also called nonFourier motion,[58] has been seen as a

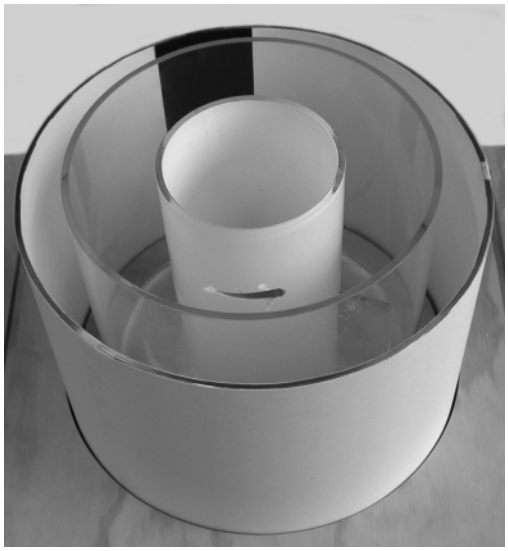

FIG. 5. Adult escape response. The fish tries to stay on the opposite site of the threatening stripe.

higher-level processing task requiring a visual cortex.

The question if animals without a cortex are nevertheless capable of using second order motion cues has been elegantly addressed in the zebrafish. By using the larval OMR paradigm, Orger et al.[59] reported that larval zebrafish display optomotor behavior in response to second-order motion stimulation. Therefore second-order motion detection does not necessitate a visual cortex and is likely extracted early in the vertebrate visual pathway.

Color vision

Different stimuli in the environment are processed by distinct channels of the visual system. Visual input is deconstructed, processed in parallel channels, and later reconstructed. For instance, in primates, distinct pathways are responsible for form and motion processing. In contrast to form vision, which uses color information, motion vision is generally color-blind, using only subsets of cone photoreceptors. Human motion perception receives stronger inputs from L and M cones as compared to S cones.[60] This is also true for a variety of other animals. Thus, prevalent usage of longer wavelength cones for motion detection is a common feature of a variety of organisms.

The OMR technique was used to assay motion perception in larval zebrafish. It was shown that inputs to motion vision derive predominantly from L (red) and M (green) cones, but not from short wavelength (UV and S (blue)) cones.[47] These findings mirror the situation in higher vertebrates. OMR responses to red–green gratings superimposed in phase are greater than either grating alone. This indicates that motion detection is dominated by a luminance channel which pools L and M cone signals, similar to human visual perception. The relative contributions of red/green cones vary with spatial frequency, indicating that inputs are spatially filtered before being pooled.[47]

Light adaptation

One of the most impressive features of the visual system is its operation range. The visual system can perceive and process visual information over a range of ten orders of magnitude of irradiance. The existence of a duplex retina, containing rod and cone photoreceptors, and a number of adaptation mechanisms form the basis of this amazing ability.

The larval zebrafish lends itself ideally for a genetic analysis of such adaptation mechanisms. Adaptation can readily be measured by the described behavioral paradigms for instance by assessing the recovery of the behavior after darkness or bright light exposure.

In such an analysis it was shown that adaptation to bright light after dark adaptation takes much longer in the tyrosinase deficient *sandy* strain.[61] The authors could show that this defect is independent of melanin synthesis (which is absent in the mutant) and postsynaptic to photoreceptors. This effect on the retinal network may be mediated by L-DOPA produced in the retinal pigment epithelium. Hence the clever usage of behavioral assays allows for the dissection of complex adaptive properties of the retinal network.

Another study highlights the advantage that the cone dominant zebrafish retina can add to the study of visual adaptation. A crucial step in desensitization of rod photoreceptors after light exposure is mediated by phosphorylation of activated rhodopsin.[62] This phosphorylation step is carried out by the rhodopsin kinase. This protein is found in both rod and cone photoreceptors in nocturnal animals such as the mouse. However, most diurnal animals lack rhodopsin kinase. Therefore this enzyme cannot be responsible for cone adaptation. Recently, it was shown that *GRK7*, another family member of the G-protein coupled receptor kinase family, is exclusively expressed in zebrafish cones but not rods. Functional inactivation by injection of morpholinos led to a pronounced delay in photoresponse recovery after bleaching.[63] Therefore, cone specific adaptation mechanisms of importance for human vision can be studied in the zebrafish but not in the mouse.

OUTLOOK

A number of behavioral methods to assess visual performance have been applied in the

zebrafish. These methods are currently used for genetic screens and for assessing visual performance following a number of genetic, pharmacological, and environmental manipulations. However, most methods currently used are based on motion cues, which bias the visual features that can be studied with these methods. Additionally, there is a need for additional behavioral assays for adult vision. We have hardly tapped into the genetics of adult vision, mainly due to the huge logistic efforts to carry out screens for recessive mutations in adult fish. The recent technical advance of generating mutations in genes of interest by the TILLING (Targeting Induced Local Lesions) method will partially alleviate this limitation and thereby aggravate the need for robust adult visual assays.[64,65] Many human ocular diseases are late onsetting and progressive and are therefore not readily modeled at larval stages. Mutant strains with mutations affecting the adult visual system will therefore be of tremendous medical interest.

Technical advances of reverse genetic tools for the zebrafish will also have a huge impact, particularly on vision research in the zebrafish. The effect of the routinely used injection of morpholino antisense nucleotides wears off at early larval stages, so any stage older than 7 dpf can not be studied by reverse genetic means. Hence, rod vision is currently not accessible by reverse genetic tools in the zebrafish. Electroporation of fluorescently labeled morpholinos into the target tissue might circumvent some of these limitations.[66,67] The development of RNA interference technology in the zebrafish may allow for the specific and long lasting downregulation of proteins in specific cells by using cell-type specific promoters.

The zebrafish offers the unique opportunity to combine genetics with fine-grained behavioral analysis of the visual system. A number of relative simple behavioral paradigms have been developed over the last couple of years. With these tools in hand we can start to genetically dissect various aspects of light perception and visual processing. The huge potential of such an approach has just been realized and will tremendously advance our knowledge about the vertebrate visual system including its allied diseases.

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