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METHOD ARTICLE

REVISED Affordable and effective optokinetic response methods to assess visual acuity and contrast sensitivity in larval to juvenile zebrafish

[version 2; peer review: 2 approved]

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Abstract

The optokinetic response (OKR) is an effective behavioural assay to investigate functional vision in zebrafish. The rapid and widespread use of gene editing, drug screening and environmental modulation technologies has resulted in a broader need for visual neuroscience researchers to access affordable and more sensitive OKR, contrast sensitivity (CS) and visual acuity (VA) assays. Here, we demonstrate how 2D- and 3D-printed, striped patterns or drums coupled with a motorised base and microscope provide a simple, cost-effective but efficient means to assay OKR, CS and VA in larval-juvenile zebrafish.

In wild-type, five days post-fertilisation (dpf) zebrafish, the 2D or 3D drums printed with the standard OKR stimulus of 0.02 cycles per degree (cpd), 100% black-white contrast evoked equivalent responses of 24.2±3.9 or 21.8±3.9 saccades per minute, respectively. Furthermore, although the OKR number was significantly reduced compared to the 0.02 cpd drum ($p<0.0001$), the 2D and 3D drums 0.06 and 0.2 cpd drums elicited equivalent responses with both 2D and 3D set-ups. Notably, standard OKRs varied with time of day; peak responses of 29.8±7 saccades per minute occurred in the early afternoon with significantly reduced responses occurring in the early morning or late afternoon (18.5±3 and 18.4±4.5 saccades per minute, respectively). A customised series of 2D printed drums enabled analysis of VA and CS in 5-21 dpf zebrafish. The saccadic frequency in VA assays was inversely proportional to age and spatial frequency and

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in CS assays was inversely proportional to age and directly proportional to contrast of the stimulus (i.e. Zebrafish evoked less saccades at less black-white contrast).

OKR, VA and CS of zebrafish larvae can be efficiently measured using 2D- or 3D-printed striped drums. For data consistency the luminance of the OKR light source, the time of day when the analysis is performed, and the order of presentation of VA and CS drums must be considered. These simple methods allow effective and more sensitive analysis of functional vision in zebrafish.

Keywords
Optokinetic response, visual acuity, spatial frequency, contrast sensitivity, visual function, zebrafish



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REVISED **Amendments from Version 1**

The authors would like to thank the reviewers for the time and effort dedicated to reviewing our paper. Version 2 contains the following changes in response to their insightful suggestions:

Clarifications regarding the results were made in Abstract

The OKR Equipment and Statistical Analysis Methods were described more precisely.

Standard deviation was included for all values described.

All experiments timepoints were included.

Justification of the use of 2D and 3D set-ups was described more precisely in the Discussion section.

Light:Dark cycle diagram of Figure 4A was modified for clarification purposes

Color format of Figure 6 and Figure 7 was changed to minimise confusion and the statistical test used was clarified in their corresponding legends.

Any further responses from the reviewers can be found at the end of the article

Plain language summary

The optokinetic response is a slow horizontal eye movement tracking an object followed by a fast movement in the opposite direction. This response can occur in humans, mammals and fish. Here, we developed simple methods to measure the optokinetic response in order to assess visual acuity and contrast sensitivity in zebrafish. In five- to 21-day-old zebrafish larvae, printed black-white or grey-white striped cylinders were rotated around the fish to induce the optokinetic response. Light levels and time of the day influence the optokinetic response. Visual acuity and contrast sensitivity can be measured simply and feasibly with printed striped cylinders from five- to 16-day-old zebrafish.

Abbreviations

AREC: Animal Research Ethics Committee; Cd/m^2: candela per square meter; Cpd: cycles per degree; Cm: centimetres; CS: contrast sensitivity; Dpf: days post-fertilization; Hpf: hours post-fertilization; OKN: optokinetic nystagmus; OKR: optokinetic response; PLA: polylactic acid; VA: visual acuity; Wt: wild type.

Introduction

The ability of researchers to effectively assess functional vision is critical to understanding the ontogeny of vision, the genetic and environmental mechanisms underlying impaired vison and the efficacy of therapeutic interventions[1,2]. The optokinetic response (OKR), or optokinetic nystagmus (OKN), is an innate behavioural response in humans[3], primates[4], mammals[5] and teleosts[6]. In clinical practice, the OKN is an objective measure of visual acuity (VA), and can be evoked by presenting moving stimuli in front of patients by changing direction or size[7,8]. In natural environments, the OKR is essential for animals to hunt, feed and avoid predators. The OKR presents as a saccadic eye movement consisting of two phases: *i)* a slow eye movement following the stimulus, in the same direction as the stimulus; and *ii)* a rapid eye movement in the opposite direction to fixate on a subsequent stimulus. These movements help to stabilise the moving image presented to the retina[9].

Here, we sought to generate simple and affordable tools for OKR assays in zebrafish and validate their efficacy in quantifying VA and contrast sensitivity (CS) in larval and juvenile zebrafish. Zebrafish are widely used to investigate the biology of vision and blindness[10]. Large clutches of embryos are readily obtained, which morphologically develop eyes within 24 hours, and by five days post-fertilisation (dpf) exhibit functional vision, including a robust OKR[11,12]. Commonly, OKR analyses in zebrafish only utilise one standard stimulus *i.e.* a drum of 0.02 cpd (*e.g.* 1 cm width stripes) and 100% black and white contrast stripes[13,14]. This is not sufficient to detect subtle impairments in vision. One approach to assessing vision more thoroughly is to vary the optokinetic stimulation. By varying the width of the stripes, VA is measured efficiently[15]. Altering the extent of contrast between stripes enables the measurement of CS[16]. Such assays have previously been successfully performed in zebrafish by specialists often using automatic or semi-automated OKR stimulators and specialised software[15–17]. However, such bespoke equipment is often inaccessible or unaffordable to many research groups. Here, we describe two simple and affordable methods to assess VA and CS in zebrafish using either 2D and 3D printed striped patterns/drums to quantify OKR, VA and CS in larval and juvenile zebrafish.

Methods

Zebrafish husbandry

All experiments using animals were approved by ethical approvals granted by the University College Dublin Animal Research Ethics Committee (AREC-14-68-Kennedy and AREC-20-12-Kennedy). Adult wild-type (*wt-Tübingen*) zebrafish were maintained in holding tanks on a 14:10 h light-dark cycle in a recirculating water system under environmental parameters averaging temperature of 28°C, conductivity of 1347 µS and pH of 7.1[18]. Adult *wt* zebrafish were fed shrimp and dry pellet food twice daily. After the noon feed, male and female adults were placed in breeding tanks and *wt* zebrafish embryos obtained by natural spawning, collected the next morning and raised in embryo medium (0.137 M NaCl, 5.4 mM KCl, 5.5 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$ with 1 ml methylene blue) until five days post-fertilisation (dpf). Larvae were fed: i) SDS 100 and paramecium from 5 to 10 dpf, ii) SDS 100, paramecium and shrimp from 11 to 20 dpf, and iii) SDS 200 and shrimp from 21 to 28 dpf. From a population of N=50 and N=40 larvae, n=12 larvae were randomly selected for VA and CS experiments at each timepoint for Protocol I and at 16 dpf for Protocol II, respectively. After each experiment, larvae were returned to the tank.

OKR equipment

A simple and affordable OKR apparatus (Figure 1) was assembled with a Nikon SMZ800 microscope (Micron Optical) to observe zebrafish eye movements; an electronic motor (RS

Radionics) connected to a non-patterned 6 cm rotating circular base in which the 2D printed striped stimulus pattern was placed (Figure 1C). A Schott KL2500 LED light source (Mason technologies) fitted with dual goose neck lightguides was positioned to illuminate inside the drum (Figure 1B). The dimensions of the 2D-printed striped patterns, generated with MS PowerPoint® and printed on stock cardboard, were 3.4 cm high and 6 cm in diameter (Figure 1D and Figure 2). The VA drums were 0.0277, 0.0416, 0.0638, 0.1111 and 0.2027 cycles per degree (cpd) were chosen based on a previous publication. For convenience, we will refer to these drums as 0.02, 0.04, 0.06, 0.1 and 0.2 cpd, respectively[15]. They were designed by changing the width of the 100% black and white contrast stripes to the calculated cycles per degree (cpd = n° of cycles/360°) when mounted on the rotating base. CS drums ranging from 100 – 20% were generated from a 0.02 cpd pattern, maintaining the spatial frequency. The black colour of the stripes was changed to include a centre to peripheral vertical gradient generated in Powerpoint™ using the "Gradient Tool, Linear Right Option" and adjusting the transparency of the Mid Gradient Stop according to the desired contrast. For example, 40% black contrast was made by changing the transparency to 60%. (C=100%-60%=40%). Additional VA drums were printed with 3D printing technology in polylactic acid (PLA), a stronger thermoplastic (Materialise UK Ltd) and placed on rotating circular base (Figure 1E). 3D drums were designed following the same parameters as 2D-printed patterns (height= 5 cm; diameter=6 cm; cpd=0.02, 0.06 and 0.2; >99% contrast).

Luminance measurement

An LS-100 luminance meter (Konica Minolta) measured, in candela per square meter (cd/m^2), the light reflected from the drum under different light intensity settings of the Schott 2500. The luminance meter was placed at 18 cm horizontally and 30 cm high from the centre of the Petri dish at 60° angle. We establish four measurements at 22.7, 12, 7 and 2%, corresponding to 3616, 1426, 769.1 and 226.7 cd/m^2.

Drum velocity

The drum base was rotated with a constant angular velocity of 100 degrees per second.

VA and CS methods (Protocols I and II)

To measure saccades/minute, a 6 cm Petri dish with 9% methylcellulose (Sigma Aldrich, UK) diluted in embryo medium was placed inside the rotating drum. From another Petri dish, larval or juvenile zebrafish in embryo medium were randomly selected and immobilised in the centre of the OKR Petri dish with 9% methylcellulose. Rotating the patterned drums 30 seconds clockwise, followed by 30 seconds counterclockwise at 100 degrees/second, evoked horizontal eye movements which were counted manually. The standard OKR utilised a drum with 0.02 cpd and 100% black-white striped contrast (Figure 4, Figure 2A). For VA assays, the OKR was performed with 2D printed patterns of 0.02, 0.04, 0.06, 0.1 and 0.2 cpd and 100% black stripe contrast for all cpd tested (Figure 5A, Figure 2A). In the CS assays, OKR was performed with 2D printed patterns of 100%, 80% 60%, 40% and 20% black/grey-white striped contrast, and 0.02 cpd all percentages (Figure 5B, Figure 2B). Drums were presented following that order, from lowest to highest spatial frequency and from highest to lowest black striped contrast. Two different protocols *(I and II)* were applied. In *Protocol I*, a zebrafish larva was randomly chosen between all larvae in the dish, placed central of the 0.02 cpd drum and saccades per

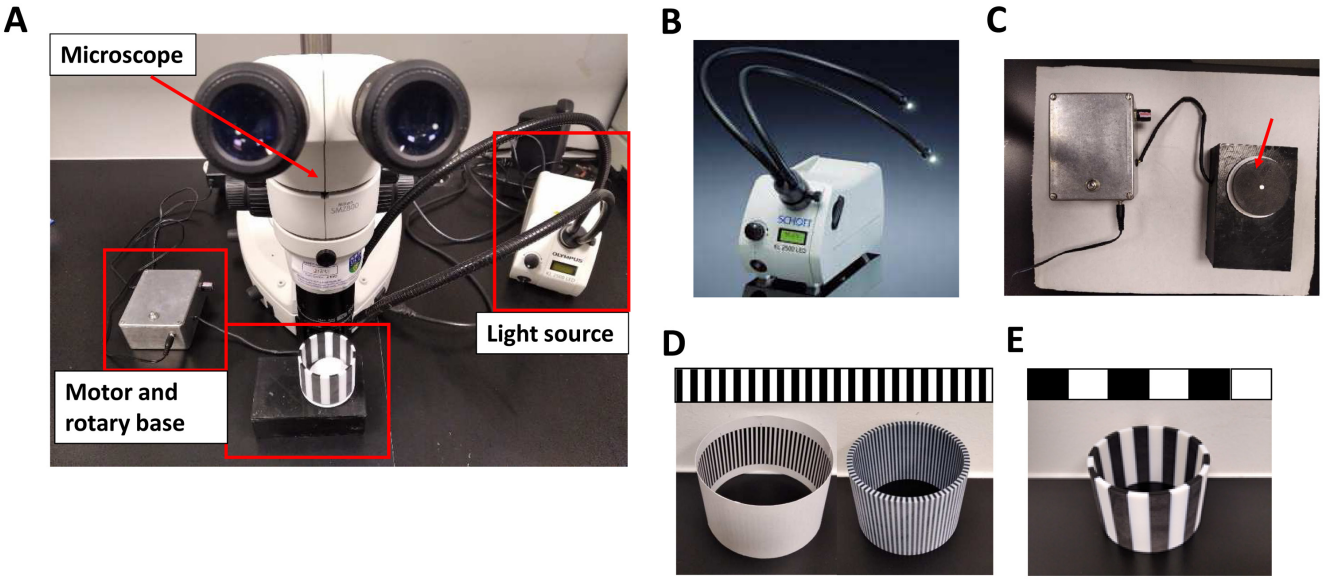
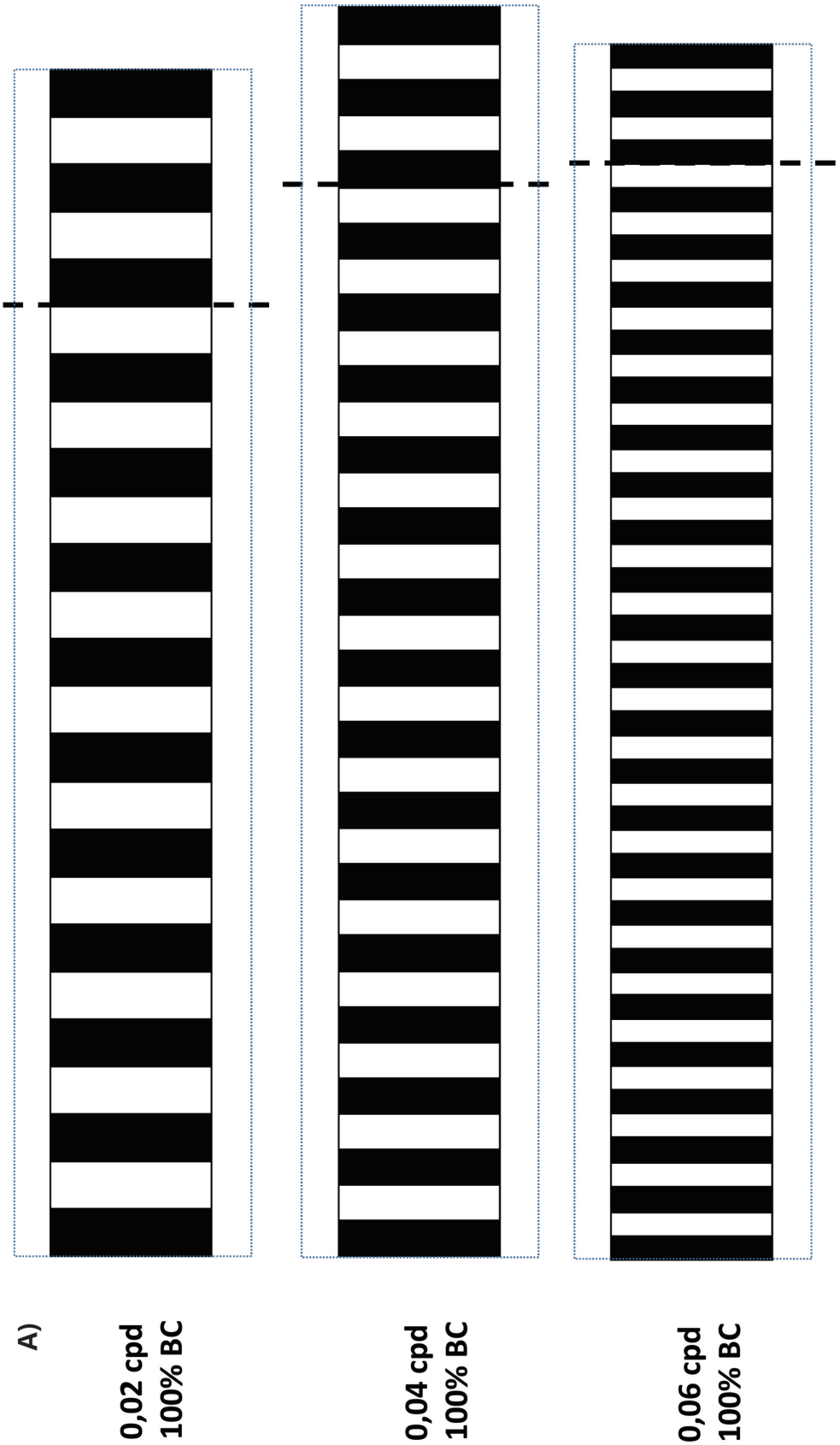
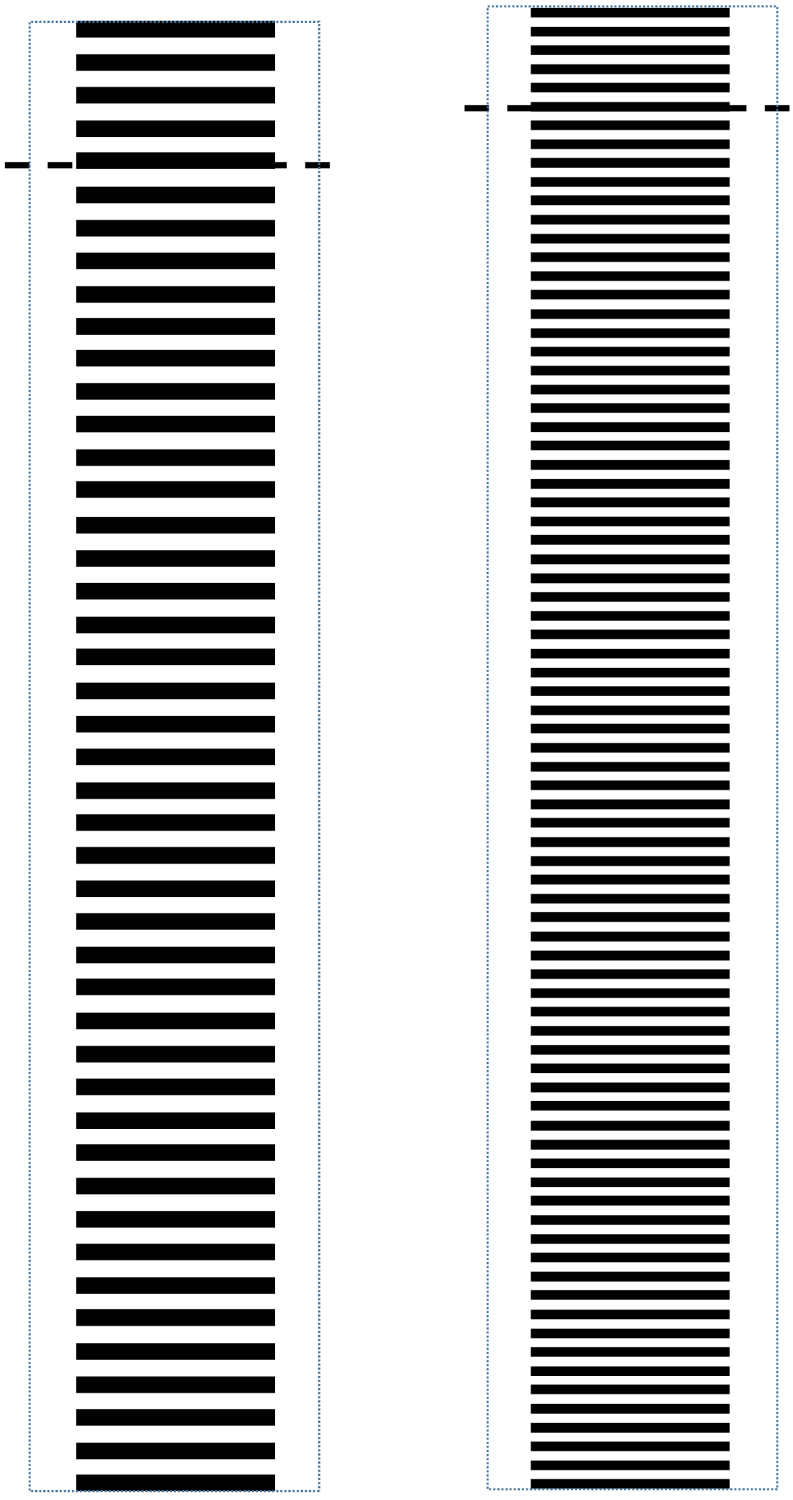


Figure 1. Optokinetic response equipment. A. Fully assembled optokinetic response apparatus. The second light guide is placed on the opposite side to the first guide to uniformly illuminate the inside of the drum. **B.** SCHOTT LED cold light source KL2500LED with fiber optic lightguide https://www.schott.com/lightingimaging/english/microscopy/index.html. Authors have obtained permission to use this image and information from SCHOTT Lighting and Imaging. **C.** Motorised rotary base to assemble the OKR drums (arrow). **D.** Optokinetic response 2D (left) and 3D (right) drums of 0.2 cycles per degree (cpd). **E.** Optokinetic response 3D drum of 0.02 (standard optokinetic response).


A)
0,02 cpd
100% BC
0,04 cpd
100% BC
0,06 cpd
100% BC


0,1 cpd
100% BC
0,2 cpd
100% BC

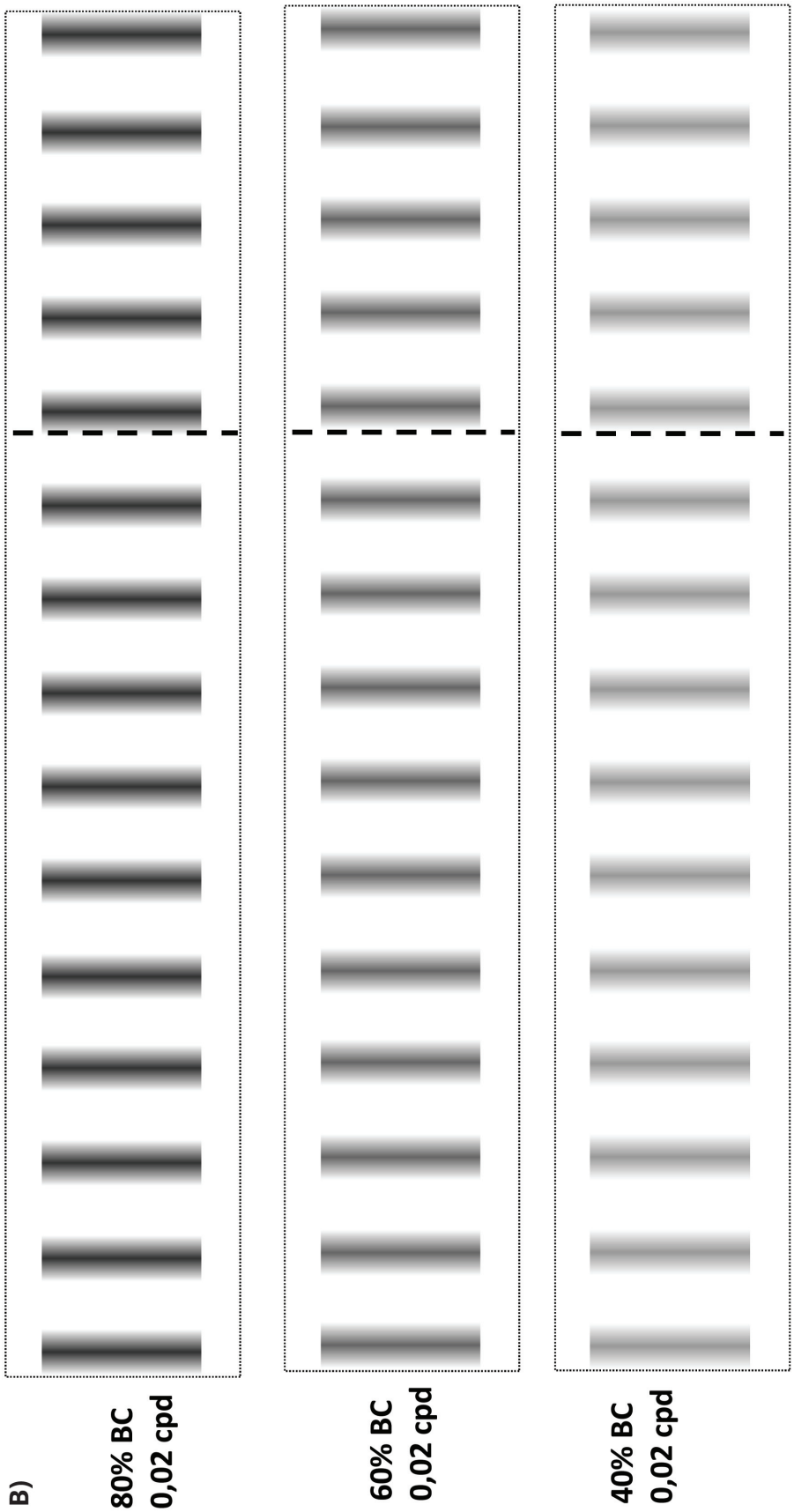

B)
80% BC
0,02 cpd
60% BC
0,02 cpd
40% BC
0,02 cpd

Figure 2. Optokinetic response 2-D visual acuity and contrast sensitivity patterns. A. 2D-printed visual acuity patterns. cpd = cycles per degree. **B**. 2D-printed contrast sensitivity patterns. bc = black contrast. All patterns were cut by dotted line and assembled by the discontinuous line to build the 2D-printed drums.

minute were counted. Subsequently and consecutively, the drum was replaced with one of higher spatial frequency (for VA) or lower contrast (CS) and saccades per minute counted. After completing one set of drums, another larva was randomly selected between all larvae in the dish and used to repeat the same drum sequence. In *Protocol II*, instead of presenting each drum of a series to the same larva, different specimens were used for each drum, i.e. larvae were naïve for OKR. In practice, a larva was analysed with 0.02 drum, saccades per minute were counted and then replaced by another larva which was subjected again to the same drum. This procedure was repeated for the rest of the drum patterns.

Statistical analysis

Statistical analysis was completed using GraphPad Prism 7.00 software (GraphPad, San Diego, CA). All saccades per minute values described in this manuscript are a group average. One-way repeated measures ANOVA was employed to determine significant differences between groups followed by Bonferroni's multiple comparisons test except for Figure 6B and Figure 7B where One-way unpaired ANOVA was used to determine significant differences between groups followed by Tukey's multiple comparisons test. Significance levels were set at $p < 0.05$.

An earlier version of this article can be found on bioRxiv (doi: https://doi.org/10.1101/2021.04.26.441419).

Results

2D and 3D printed VA patterns elicit equivalent OKR responses in 5 dpf zebrafish

The manual OKR equipment set-up (Figure 1) permits simple exchange of stimulus patterns to measure VA. This apparatus was assembled using a microscope (Figure 1A) to observe zebrafish eye movements, a light source (Figure 1B) and an electronic motor connected to a 6 cm rotating circular base (Figure 1C). 2D or 3D printed stimulus drums (Figure 1D) were placed on the circular base which was rotated electronically to evoke eye movements. A standard OKR pattern of 0.02 cpd (100% contrast) (Figure 1E) and customised 0.06 and 0.2 cpd patterned stimuli (Figure 1D) were produced by 2D or 3D printing (see Methods and Figure 2 for full details on OKR assembly).

VA analysis with 2D and 3D printed drums was performed on 5 dpf zebrafish larvae (~123.5 hours post-fertilisation - hpf) using *Protocol I* (see Methods) (Figure 3). The OKRs evoked by the 3D and 2D-printed drums were equivalent. More specifically, the OKR activity with the 0.02 cpd 2D-printed pattern (24.2±3.9 saccades per minute) was equivalent to the 21.8±3.9 saccades per minute evoked with the 0.02 cpd 3D-printed drum (Figure 3). Similarly, there was no significant difference between the 7.5±2.9 and 5.3±3.9 saccades per minute produced by the 0.06 cpd 2D and 3D-printed drum, respectively (Figure 3). At the

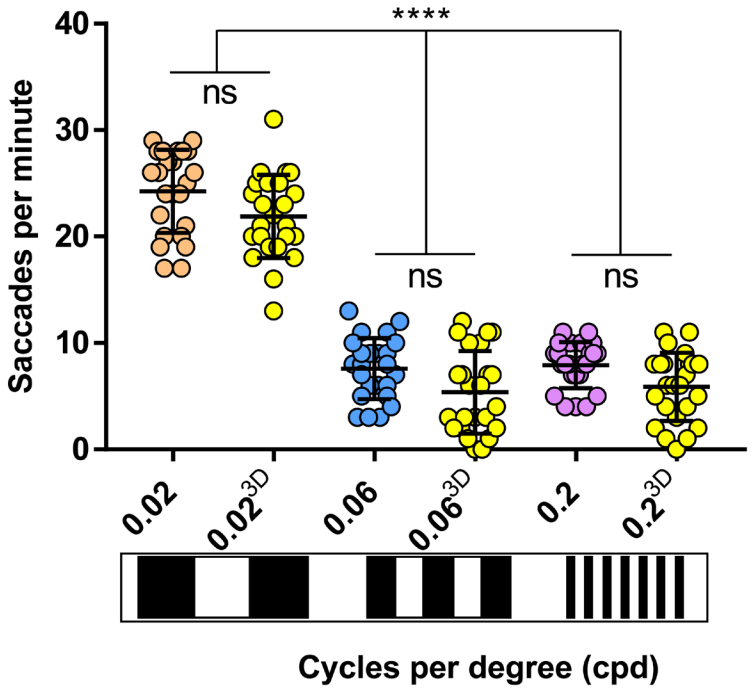


Figure 3. 2D drums evoke same saccades frequency as 3D drums. Visual acuity responses obtained with 3D drums (yellow dots) don't vary with respect of cardboard-printed drums (0.02 cpd, orange dots; 0.06 cpd, blue dots; 0.2 cpd, purple dots). 0.06 and 0.2 cpd responses evoked with 2D and 3D-printed drums were significant lower than standard optokinetic response activity (0.02 cpd) with 2D and 3D-printed drums. Data were analyzed by one way ANOVA and Bonferroni's multiple comparison test, where ns is no significant difference ($p>0.05$) and ****=$p<0.0001$. Error bars indicate standard deviation. Midline of error bars represents the group average. Three replicates of eight larvae per drum, n=24.

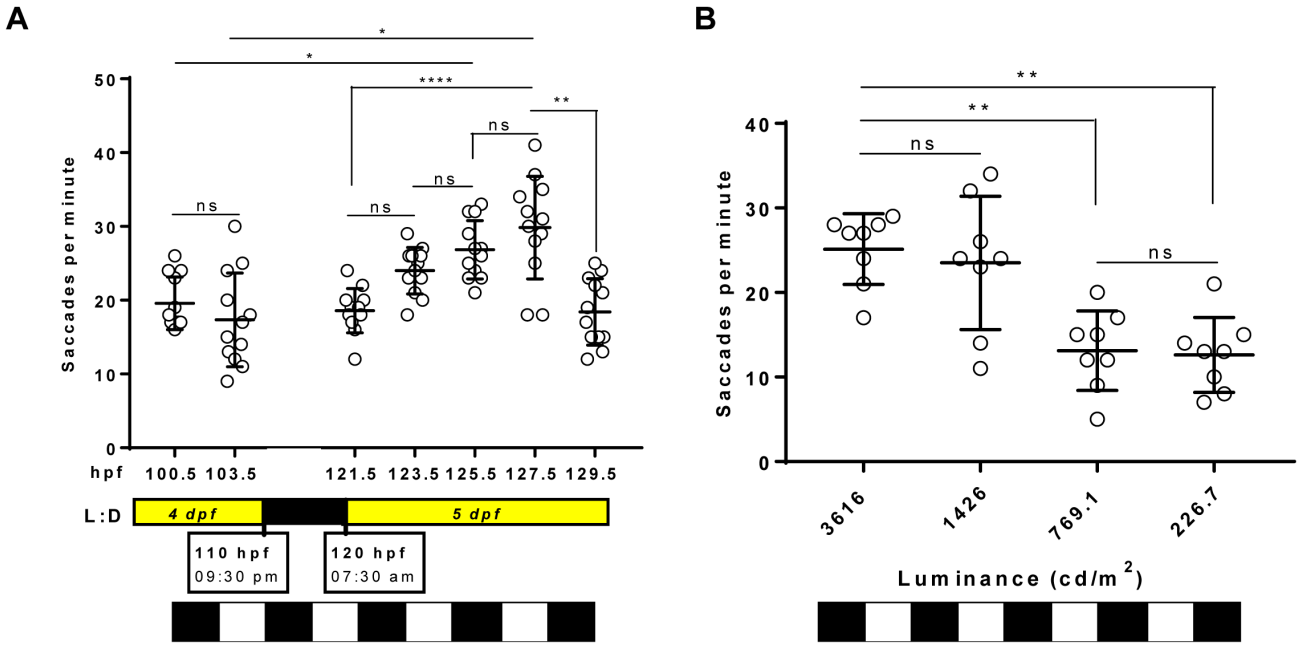


Figure 4. Optokinetic response (OKR) is modulated by time of day and luminance levels. A. Standard OKR (0.02 cycles per degree [cpd], 100% black contrast, 3616 candelas per square meter [cd/m^2]) at different timepoints along 4 and 5 days post fertilisation (dpf). Equivalent times (100.5 vs. 125.5 hpf; 103 vs. 127.5 hpf) show an increase in OKR between 4 and 5 dpf. Highest OKR yields at 127.5 hpf. **B.** Standard OKR (0.02 cpd, 100% black contrast) at different levels of luminance at 125 hpf. Higher levels of luminance evoked a better response on zebrafish but at 1426 cd/m2, OKR response is more variable (SD=7.8 saccades per minute) than 3616 cd/m2 (SD=4.1 saccades per minute). Data were analyzed by one-way ANOVA and Bonferroni's multiple comparison tests, where ns is no significant difference, ($p>0.05$), **=$p<0.01$ and ****=$p<0.0001$. Error bars indicate standard deviation (SD). Midline of error bars represents the group average. One replicate of 12 larvae per timepoint, n=12.

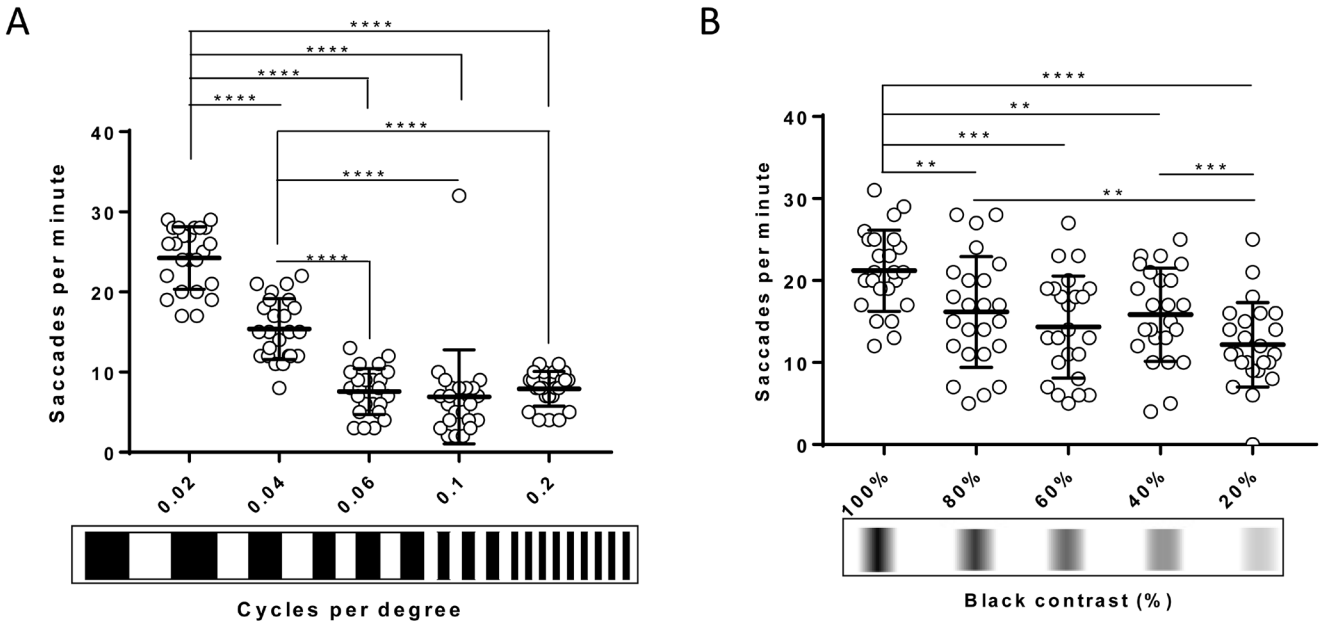


Figure 5. The 2D-printed drums enable discrimination of visual acuity and contrast sensitivity in larval zebrafish. A. Visual acuity of 5 dpf wild-type zebrafish larvae decreases progressively when width of the stripes is reduced compared to standard optokinetic response (OKR) and 0.04 cpd. From 0.06 cpd, response is constant. **B.** Contrast sensitivity of 5 dpf wild-type zebrafish larvae decreases slowly when contrast between black-white stripes is lowered compared to standard OKR. 20% black contrast evokes the lowest response. Data were analyzed by repeated measures one-way ANOVA and Bonferroni's multiple comparison test, where ns is no significant difference, ($p>0.05$), **=$p<0.01$, ***=$p<0.001$ and ****=$p<0.0001$. Error bars indicate standard deviation. Midline of error bars represents the group average. Three replicates of eight larvae, n=24 measurements per pattern.

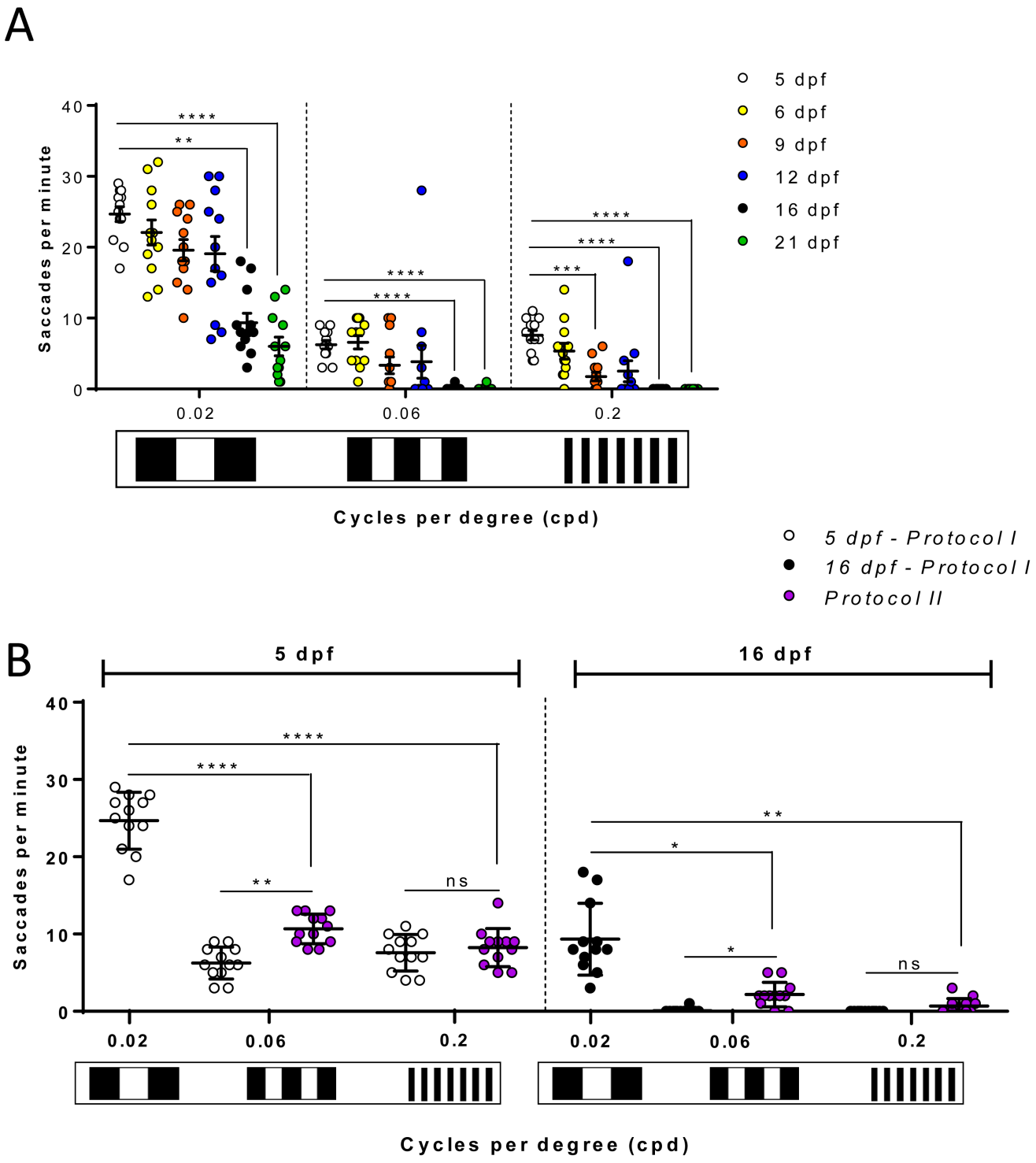


Figure 6. Zebrafish visual acuity responses show age-dependent variations. A. Visual acuity of zebrafish from 5 to 21 days post fertlisation (dpf) drops significantly from 16 dpf following *Protocol I* at 0.02, 0.06 and 0.2 cpd. At 0.2 cpd, this decreased response is also remarkable on 9 dpf. One replicate of 12 larvae per each set of patterns. **B.** Comparison of visual acuities measured with *Protocol I* (black dots) and *Protocol II* (red dots) on 5 dpf and 16 dpf. 0.02 cpd responses at 5 dpf (purple dots) and 16 dpf (white dots) belong to *Protocol I* and *Protocol II* as it is the first pattern tested. There is no difference between both protocols except at 0.06 cpd where 5 dpf naïve larvae showed a higher number of saccades. Data were analyzed by repeated measures one-way ANOVA and Bonferroni's multiple comparison test in **A** and unpaired measures one-way ANOVA and Tukey's multiple comparison test in **B**. In both statistical tests, ns is no significant difference, (p>0.05), **=p<0.01, ***=p<0.001 and ****=p<0.0001. Error bars indicate standard error of mean in **A** and standard deviation in **B**. Midline of error bars represents the group average. One replicate of 12 independent larvae for each pattern, n=12.

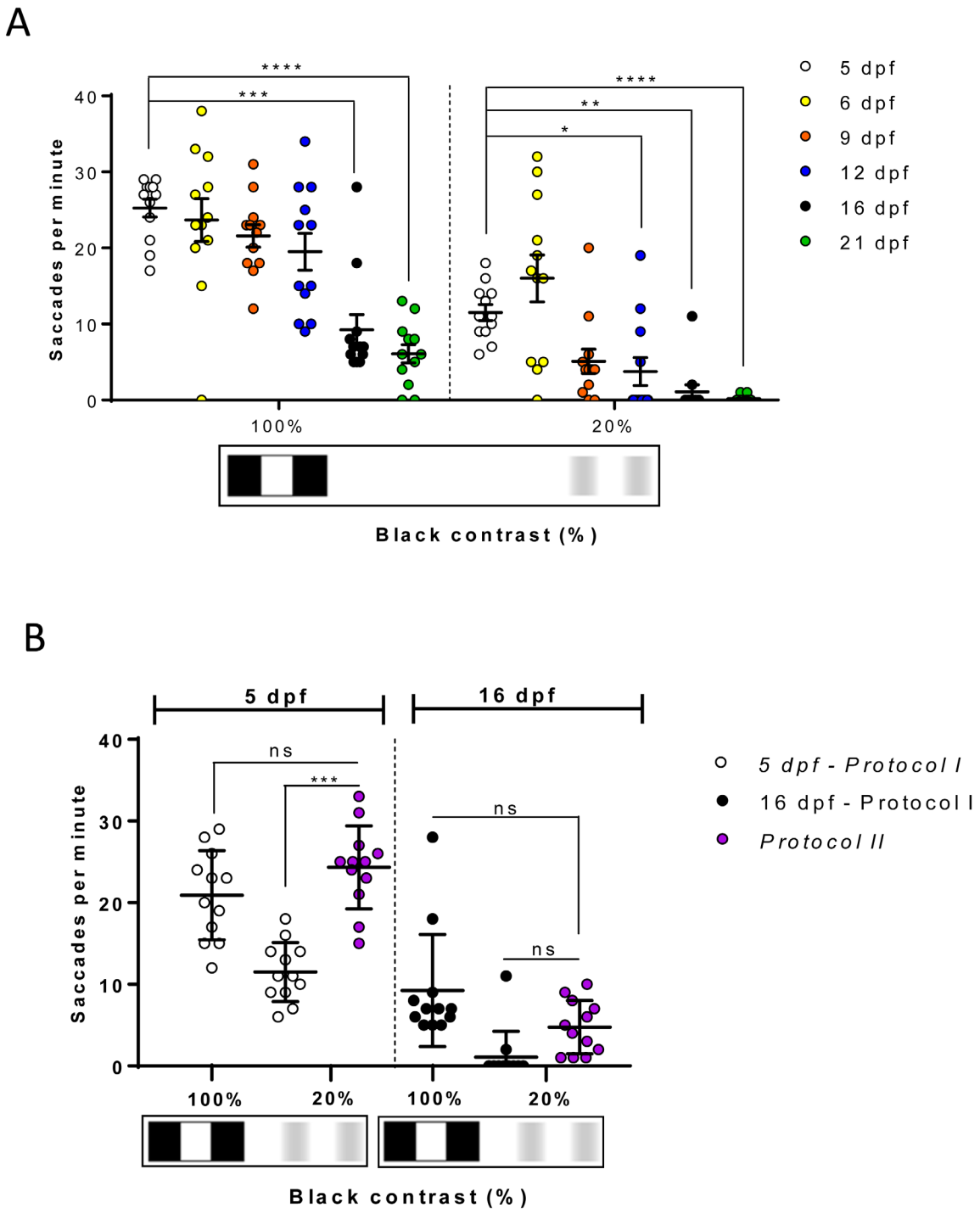


Figure 7. The contrast sensitivity response of juvenile zebrafish diminishes with age. A. Optokinetic response to 20% is significant at 12, 16 and 21 days post fertilisation (dpf). **B.** Comparison of contrast sensitivity responses measured with *Protocol I* (black dots) and *Protocol II* (red dots). 100% black contrast at 5 dpf (purple dots) and 16 dpf (white dots) belong to *Protocol I* and *Protocol II* as it is the first pattern tested. Responses of 20% black contrast with *Protocol II* are higher than when evoked with *Protocol I*. However, there are no differences between both protocols at 16 dpf. Data were analyzed by repeated measures one-way ANOVA and Bonferroni's multiple comparison test in **A** and unpaired measures one-way ANOVA and Tukey's multiple comparison test in **B**. In both statistical tests, ns is no significant difference, ($p>0.05$), **=$p<0.01$, ***=$p<0.001$ and ****=$p<0.0001$. Error bars indicate standard error of mean in **A** and standard deviation in **B**. Midline of error bars represents the group average. One replicate of 12 independent larvae for each pattern, n=12.

highest spatial frequency tested, 0.2 cpd, the number of saccades evoked by the 2D (7.9±2.2 saccades per minute) and 3D (5.8±3.2 saccades per minute) drums also showed no significant difference. Therefore, both 2D and 3D printed drums can be used to measure the VA of 5 dpf zebrafish larvae, the 3D-printed drums offering a more durable, but more costly option.

The zebrafish larval OKR response is modulated by time of day and luminance levels

To determine if the zebrafish larval OKR has diurnal variations, the number of saccades generated with the standard 3D-printed OKR drum (0.02 cpd) was determined at seven time-points distributed throughout the light phases of the standard 14-hour light: 10-hour dark cycle (Figure 4A). At 5 dpf, the trend observed was an increasing number of saccades until the afternoon with a subsequent drop in response (Figure 4A). The highest OKR (29.8±7 saccades per minute) was observed at early afternoon/127.5 hpf, which was significantly higher (p=0.0001) than the OKRs observed at early morning/121.5 hpf (18.5±3 saccades per minute) or at late afternoon/129.5 hpf (18.4±4.5 saccades per minute). The midday and early afternoon responses on 5 dpf (125.5 and 127.5 hpf, respectively) were significantly greater than the corresponding time of day responses at 4 dpf (100.5 and 103.5 hpf, respectively).

To evaluate if the 5 dpf OKR behaviour varied with brightness intensities, the standard OKR was assessed under luminance ranging from 226.7–3616 cd/m^2 (Figure 4B) at 125 hpf. The largest OKR activity occurred at 3616 and 1426 cd/m^2 (25.1±4.2 and 23.5±7.9 saccades per minute, respectively). The responses at 769.1 and 226.7 cd/m^2 (13.1±4.7 and 12.6±4.4 saccades per minute, respectively) were significantly lower (p=0.0081 and p=0.0035, respectively) than at 3616 cd/m^2. In summary, the larval OKR shows response variations based on time of day recorded and light intensity used.

The 2D/3D-printed striped patterns enable discrimination of VA and CS in larval zebrafish

Establishment of affordable VA and CS assays offers researchers the potential to identify more subtle defects in zebrafish vision than using standard OKR drums. Thus, bespoke 2D-printed striped patterns of 0.04 and 0.1 cpd for VA were generated (see Methods for details) and tested (Figure 5). At 123 hpf, using *Protocol I* (see Methods), an increased number of stripes reduced the number of saccades per minute, but robust and reproducible responses were observed at each cpd tested (Figure 5A). At 0.04 cpd, the OKR activity (15.3±3.8 saccades/minute) was significantly (p<0.0001) lower compared to the standard OKR pattern of 0.02 cpd (24.2±3.9 saccades per minute), but significantly higher than the response with the 0.06 cpd pattern (7.6±2.9 saccades per minute, p<0.0001). The average saccades per minute with the 0.06 cpd pattern (7.6±2.9 saccades per minute) is similar to the 0.1 and 0.2 cpd pattern (6.9±5.9 and 7.9±2.2 saccades per minute, respectively).

CS assays were also performed using 2D printed drums and *Protocol I* at 125 hpf (Figure 5B). The OKR activity evoked by the 0.02 cpd patterns with decreasing contrast was significantly reduced (80%, p=0.0022; 60%, p=0.0001; 40%, p=0.004, and 20%, p<0.0001) compared to the standard OKR drum of 0.02 cpd and 100% contrast. For example, at 80% black-white contrast, the 16.1±6.7 saccades per minute were significantly lower (p=0.0022) than the 21.2±4.9 saccades per minute evoked with the standard OKR drum pattern (0.02 cpd). There were no significant differences in response between the 80% contrast pattern and the 60% or 40% contrast pattern. The response from the 20% contrast pattern (12.2±5.1 saccades per minute) was significantly lower than with the 80% and 40% contrast pattern (p=0.0091 and p=0.0003, respectively. In summary, the 2D-printed patterns provide a simple and affordable method to assess CS and VA assays in zebrafish larvae.

The zebrafish VA response shows age-dependent variations

Using the 2D-printed patterns, we determined if the OKR-based VA response varies with age in larval to juvenile zebrafish aged 6, 9, 12, 16 or 21 dpf at 123 hpf. Interestingly, with *Protocol I* the measured VA responses decreased with age (Figure 6A) for all tested patterns. The largest OKR of 24.6±3.7 saccades per minute was achieved at 5 dpf with a pattern of 0.02 cpd frequency (Figure 6A). The lowest OKR, with absence of any saccadic eye movements (0±0 saccades per minute), was obtained with 16 and 21 dpf zebrafish using patterns of 0.2 cpd (Figure 6A). With patterns of 0.02 cpd, the OKR was significantly reduced at 16 dpf (p=0.0016) and 21 dpf (p<0.0001) compared to 5 dpf larvae, with 62% and 76% reductions in eye saccades, respectively. With patterns of 0.06 cpd, the highest responses were observed at 5 and 6 dpf (6.2±2.1 and 6.5±3.2 saccades per minute, respectively), which significantly declined at 16 dpf (0.1±0.3 saccades per minute, p<0.0001) and 21 dpf (0.1±0.3 saccades per minute, p<0.0001) compared to 5 dpf. For the highest VA patterns tested (0.2 cpd, with highest number of stripes), the largest OKR was observed in 5 dpf larvae (7.5±2.4 saccades per minute) and significantly reduced responses were observed in 9 (1.7±2.1 saccades per minute, p=0.0004), 16 (0±0 saccades per minute, p<0.0001) and 21 (0±0 saccades per minute, p<0.0001) dpf zebrafish. Note that at 16 dpf, when responses to VA and CS drums dropped, fish immobilisation in methylcellulose during drum stimulation was more difficult compared to earlier stages. In addition to observing an age-dependent reduction in OKR at each cpd frequency, we also observed that the level of response with the 0.06 and 0.2 cpd patterns were much lower than with the 0.02 cpd standard drum (Figure 6A). In *Protocol I,* the data is generated based on first testing larvae at the lowest spatial frequency, and subsequent testing in the next higher spatial frequency drum. Therefore, to assess whether the reduction in OKR with drums of higher spatial frequency was due to adaptation to previous OKR stimuli, we repeated the assays on 5 and 16 dpf at 123 hpf, using *Protocol II* (see Methods for details) where each fish was tested with only one drum pattern (Figure 6B). In Figure 6b, datasets from 5 and 16 dpf groups for Protocol I are from the same groups in Figure 6a. In 5 dpf zebrafish, there was no significant difference in OKR using *Protocol I* or *II* for 0.2 cpd pattern (Figure 6B). There was a significant increase (p=0.0017) in OKR of 5 dpf larvae with *Protocol II* compared to *Protocol I* with the 0.06 cpd pattern (Figure 6B). However, the *Protocol II* response of 10.6±1.9 saccades per minute with the 0.06 cpd pattern was still

significantly lower ($p<0.0001$) than the 24.6±3.7 saccades per minute observed under *Protocol I* with the 0.02 cpd standard drum (Figure 6B). In 16 dpf zebrafish, a slight but significant increase ($p=0.044$) in OKR was noticed when *Protocol II* is compared to *Protocol I* with the 0.06 cpd pattern. With the 0.2 cpd pattern and 16 dpf zebrafish there was no significant difference using *Protocol I* or *Protocol II*. At 16 dpf, the 0.06 cpd response obtained with *Protocol II* (2.1±1.6 saccades per minute) is significantly lower ($p=0.0204$) than the 0.02 cpd response (9.3±4.6 saccades per minute). In summary, all the above suggests that VA measurements drop after 12 dpf. Additionally, care needs to be taken regarding a consistent order of testing the VA drums to avoid experimental artifacts.

The zebrafish CS responses show age-dependent variations

Subsequently, we determined if the CS responses obtained using the 2D printed patterns displayed age-dependent variations. At 125 hpf, using *Protocol I* and 0.02 cpd drums with 100% black-white contrast, the largest response of 25.2±4.1 saccades per minute was observed with 5 dpf larval zebrafish (Figure 7A). Responses to these drums showed significant reduction with age, but reproducible visual behaviour responses were still observed with 16 and 21 dpf juvenile zebrafish (9.2±6.9 saccades per minute, $p=0.0007$; and 6±4.2 saccades per minute, $p<0.0001$, respectively). Similarly, with the 20% contrast drums, the largest responses were observed with 5 and 6 dpf (11.5±3.6 and 16±10.7 saccades per minute, respectively) larvae. Numbers declined with age and significant reductions were observed in 12, 16 and 21 dpf juveniles (3.7±6.3 saccades per minute, $p=0.04$; 1.1±3.2 saccadic per minute, $p=0.0019$; 0.1±0.4 saccades per minute, $p<0.0001$, respectively). As mentioned earlier, fish immobilisation and saccade counting in older fish is less consistent. Again, at 125 hpf, we utilised *Protocol II* to determine if reduced responses were due to desensitisation to consecutive stimuli. In Figure 7B, datasets from 5 and 16 dpf groups for *Protocol I* are from the same groups in Figure 7A. In 16 dpf zebrafish, there was no significant difference in OKR using *Protocol I or II* when testing 20% contrast drums (Figure 7B). In 5 dpf zebrafish, there was a significant increase ($p=0.0002$) in OKR at 20% contrast when *Protocol II* is compared to *Protocol I* (Figure 7B). Indeed, the *Protocol II* response of 24.3±5.1 saccades per minute with the 20% contrast drum is equivalent to the response observed under *Protocol I* with 100% contrast (Figure 7B), suggesting the diminished CS response is due to desensitisation. In summary, our data suggests that CS responses decrease significantly after 9 dpf, and highlight the importance of strict consistency to be taken while testing different CS patterns on the fish to avoid confounding.

Discussion

Affordability and accessibility

The OKR is a strong, innate visual behaviour that is very useful to characterise functional vision in zebrafish[12]. We employed 2D and 3D printed patterns, of different stripe width or different black-white contrast, to effectively and affordably assay OKR, VA and CS in 5 to 21 dpf zebrafish. The remaining equipment required is accessible and affordable as suitable microscopes are commonly available in laboratories and the other components *e.g.* motor, light source and 2D/3D-printed patterns can be acquired easily and cost-effectively. While 2D-printed can be easily printed on cardboard, they are less durable so can be used for pilot experiments. However, if researchers prefer a more durable option, 3D-printed drums are still an affordable option (less than 100€ per drum) yielding equivalent results as 2D-printed drums. Whilst automated or computerised devices were previously used to report OKRs, those systems have high costs (up to €30,000), prohibitive to many research groups. Furthermore, computerised measurements of OKR, VA and CS, apply software to disaggregate the collective saccadic eye movement into eye velocity, gain or amplitude parameters[6,15,16]. This requires establishment of thresholds based on algorithms and formulas using specialist programmes[6,15]. In summary, the manual OKR set-up described here enables refined and accurate evaluation of OKR, VA and CS in zebrafish larvae, it is easy to use, does not require specialist software and is up to 10 times more affordable.

Effectiveness and sensitivity

With the 2D and 3D printed patterns, the magnitude of the 5 dpf VA response progressively decreased from the 0.02 cpd (standard OKR) to the 0.2 cpd (finest stripe width tested) pattern. This inverse relationship between saccadic response and stripe width agrees with previous studies using digitalised OKR set-ups and a 0.02 – 0.2 cpd range of VA patterns[15,16]. Notably, those studies, which pre-stimulated the larvae with a 0.06 cpd pattern before testing, reported 0.16 cpd as the highest VA pattern to evoke an OKR in 5 dpf larvae[15,16]. However, with our 2D and 3D printed drums, an even finer VA stimulus of 0.2 cpd elicits reproducible OKRs of 5.8±3.2 – 7.9±2.2 saccades per minute, providing enhanced ability to identify more subtle visual impairment phenotypes.

Diurnal variability

There is clear evidence of dynamic anatomical and behavioural development of zebrafish vision up to 5 dpf[9,19–21]. A previous analysis of diurnal variations in OKR at 5 dpf[22,23] showed no difference in the number of saccades evoked during the day at 122 hpf (early morning; 27 saccades per minute) and 134 hpf (early evening; 25 saccades per minute), but dropping to 0 saccades per minute at 137 hpf (night)[22]. Another study performed diurnal OKR analysis at different timepoints[23], wherein at 125 hpf the OKR gain peaked and then decreased progressively at 129 and 133 hpf. Here, we investigate the OKR between 4 and 5 dpf using even shorter time intervals. We found a cyclic modulation of OKR activity from a base of 19.5±3.6 saccades per minute at 100.5 hpf (midday) at 4 dpf, reaching a peak of 29.8±7 saccades per minute at 127.5 hpf (early afternoon) on 5 dpf and then troughing at 18.4±4.5 saccades per minute at 129.5 hpf (late afternoon) on 5 dpf. The peak responses at 125.5 and 127.5 hpf (26.8±4 and 29.5±7 saccades per minute) and diminished response at 129.5 hpf (18.4±4.5 saccades per minute) are consistent with Huang *et al.*[23]. This diurnal variation may be attributed to circadian rhythms that drive diurnal and nocturnal behaviours in zebrafish[23,24]. In summary, a more extensive characterization at shorter times post-fertilization

demonstrates significant diurnal variations in the OKR and highlights the importance of carefully controlling the time of day when OKR analysis is performed.

Light variability

OKR gain, the ratio between eye velocity and stimulus velocity during the slow saccadic phase, was previously reported to increase with luminance from 0.38 cd/m^2 up to 388 cd/m^2 levels[16]. Here, we demonstrate that higher luminance levels of 769.1 to 3616 cd/m^2 increase the saccadic frequency from 13.1±4.7 to 25.1±4.2 saccades per minute. Notably, we did not, as in previous studies, measure the luminance from where the stimulus was projected[16]. Instead our luminance was measured at the position of the fish in the methylcellulose to measure the ambient illumination surrounding the fish more accurately. In summary, the luminance of the light source must be measured and controlled during all analyses to avoid this confounding variable which affects saccadic frequency.

Contrast sensitivity and visual acuity detection

Previous VA studies on 5 dpf larvae report that the magnitude of the OKR gain or eye velocity between 0.02 and 0.2 cpd was indirectly proportional to spatial frequency[15,16]. More specifically, an eye velocity of 4 degrees/second at 0.05 cpd reduced to 0 degrees/second (no eye movements) at 0.2 cpd[12]. At the highest drum velocity tested (22.5 degrees per second) a gain response of 0.2 (max. gain=1) at 0.02 cpd decreased to 0.025 at 0.16 cpd; however, the gain peak of 0.3 was reported at a mid-frequency of 0.06 cpd[16]. The VA responses with the 2D printed patterns concur with this spatial frequency-dependency as evidenced by 24.2±3.9 saccades per minute at 0.02 cpd reducing to 7.9±2.2 saccades per minute at 0.2 cpd, the latter response contrasting with no eye movements using the computerised OKR hardware. Thus, the OKR set-up described here emulates VA responses of automatic devices[15,16] and furthermore, it detects quantifiable responses at higher spatial frequencies[12,13].

CS analysis using the 2D-printed drums (20-100%) at 5 dpf show a similar trend, as previously reported with computerised set-ups (0.7 to 100% contrast). A higher number of OKR saccades or greater OKR gain is observed as the black-white contrast increases[16,25]. Notably, these computerised devices reported a low gain[16] and no eye movements[25] at 20% black-white contrast. However, our manual OKR set-up evokes reproducible OKR saccades of 12.1±5.1 per minute at the 20% black-white contrast. Hence, our affordable 2D-printed drums can elicit OKR that discriminate higher VA frequencies and lower black-white contrast, enabling more sensitive detection of VA and CS in 5 dpf zebrafish.

Age variability

OKR analysis in juvenile zebrafish older than 5 dpf was previously reported using computerised OKR set-ups[6,12,16]. Orger *et al.*[12] used a lower drum velocity (10 degrees per second) and described the standard OKR activity at 7 dpf, showing robust eye saccades through a motion detection OKR. Beck *et al.*[6] investigating the OKR phases from 5 to 35 dpf, found that at 50 degrees per second drum velocity, gain decreased in all tested ages (5 to 35 dpf). Here, we use the 2D-printed drums to describe the saccadic frequency of 5 to 21 dpf zebrafish based on spatial frequency (Figure 6A). For all ages, saccadic frequency decreased as spatial frequency increased. We obtained quantifiable responses at all tested ages except at 16 and 21 dpf using our 0.06 and 0.2 cpd patterns. This reduction when zebrafish were older, was also observed using 100% and 20% black-white contrast 2D-printed patterns in 5 to 21 dpf zebrafish (Figure 7A). At 6 dpf, Rinner *et al.*[16] previously reported that OKR gain with 100% black-white contrast was approximately 0.7, decreasing to 0.3 with 20% black-white contrast. This response is similar to what we obtained at 6 dpf using the 2D-printed patterns, where 23.6±9.8 saccades per minute were obtained at 100% black-white contrast, decreasing to 16±10.7 saccades per minute at 20% black-white contrast. In summary, our data suggests that manual VA/CS analysis, using 2D-printed patterns, can be used to detect spatial frequency and contrast discrimination by zebrafish larvae at 6, 9, 12, 16 and 21 dpf.

Protocol variability/desensitisation

The significant drop of VA and CS response observed after 16 dpf may be explained by the use of methylcellulose to immobilise the larvae, but which is also reported to hamper oxygen exchange in zebrafish older than 7 dpf and to decrease the OKR gain[9].

Studies on adult zebrafish, aged between 4–16[26] and 12–24[17] months, placed the fish further from the stimulus, *i.e.* 7.3 cm[26] and 19.5[17] cm *versus* the 3 cm used here. According to the VA concept and VA examinations in children[27], the eye to stimulus distance should be increased with age, which suggests that 16 dpf could be a *"key"* time-point to increase the distance stimulus-eye in zebrafish.

We considered that habituation could also account for reduced VA/CS at older stages. However, using 16 dpf naïve larvae (*Protocol II*), responses at highest spatial frequencies (0.2 cpd) and lowest contrast (20% black-white contrast) were similar as those tested in 16 dpf in *Protocol I*. Our overall interpretation is that, in general, *Protocol I* is more suitable to conduct VA/CS studies in zebrafish larvae due to ethical considerations to reduce the number of individuals used, while enabling follow-up of VA or CS multiple data obtained from a single specimen. *Protocol II* could be more suitable, however, if obtaining maximum responses at 5 dpf is relevant for the study.

Conclusions

The OKR set-up described here can be easily and cost-effectively acquired to measure OKR. Our 2D-printed patterns can reliably and feasibly quantify VA and CS response in zebrafish larvae from 5 to 16 dpf. The age of the fish used, the time of the day the assay performed, the light levels within the fish position and pre-stimulation can vary the OKR and must be accurately determined for a consistent OKR, VA and CS analysis. The 2D/3D drums and methods described here can be utilised to identify and characterise more effectively zebrafish models with visual deficits.

Data availability

Underlying data

Zenodo: Affordable and Effective Optokinetic Response Methods to Assess Visual Acuity and Contrast Sensitivity in Larval to Juvenile Zebrafish. https://doi.org/10.5281/zenodo.5146212[28].

This project contains the following underlying data:

- Fig. 3 - 2D drums evoke same saccades frequency as 3D drums.csv
- Fig. 4A - OKR Response is Modulated by Time of Day.csv
- Fig. 4B - OKR Response is Modulated by Luminance Levels.csv
- Fig. 5A - The 2D-printed Drums Enable Discrimination of Visual Acuity in Larval Zebrafish.csv
- Fig. 5B - The 2D-printed Drums Enable Discrimination of Contrast Sensitivity in Larval Zebrafish.csv
- Fig. 6A - The Zebrafish Visual Acuity Responses Shows Age-Dependent Variations.csv
- Fig. 6B- The Zebrafish Visual Acuity Responses Shows Age-Dependent Variations.csv
- Fig. 7A - The Contrast Sensitivity Response of Juvenile Zebrafish Diminish with Age.csv
- Fig. 7B - The Contrast Sensitivity Response of Juvenile Zebrafish Diminish with Age.csv

Reporting guidelines

Zenodo: ARRIVE checklist for "Affordable and effective optokinetic response methods to assess visual acuity and contrast sensitivity in larval to juvenile zebrafish". https://doi.org/10.5281/zenodo.5118706[29].

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

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http://www.doi.org/10.5281/zenodo.5118706

Open Peer Review

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Version 2

Reviewer Report 11 January 2022

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✔ **Logan Ganzen**
UC Berkeley, Berkeley, CA, USA

The authors have sufficiently addressed my proposed concerns in this revision.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of research is generally in visual neuroscience. My doctoral research involved the development of zebrafish behavioral assays to perform drug screening on zebrafish models of disease.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 22 November 2021

https://doi.org/10.21956/openreseurope.15007.r28060

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✔ **Ralph Nelson**
National Taiwan University, Bethesda, MD, USA

Rotating striped drums are not a new technique to evoke an optokinetic response (OKR) from zebrafish eyes. The reviewer's first encounter with this technique was Brokerhoff *et al.* (1995) '*A behavioral screen for isolating zebrafish mutants with visual system defects*'. This design remains quite viable. This article provides a lot of details enabling labs to set up such a system, inexpensively as the authors emphasize. The article also gives a broad range of constraints to consider in experimental design. The reviewer was particularly enlightened by the studies of circadian rhythm. The larvae are most responsive at midday or early afternoon. Older larvae don't perform well in these protocols. The authors are happy to point out that the inexpensive rotating-drum stimulus was significantly more effective than expensive computer-driven displays at evoking OKR responses and extended the range of contrasts and spatial frequencies that could be examined. The reviewer wants to add that in his lab one of his students had little trouble making her own computer driven OKR stimulus for zebrafish larvae. This was a laptop, one that folds out flat, with rotating pinwheels generated by PsychoPy toolbox libraries. The larvae were placed in a petri dish on top of the monitor, and movies of the eye movements taken with an iPhone. The latter is not inexpensive, but at least little added expense (Deveau *et al.*, 2020). The reviewer expects the parametric properties of a laptop monitor as seen from below by the larvae don't equal those of a high-contrast drum seen from all sides, but they were good enough to identify color-vision mutations.

Minor points.

Abstract:

- Reviewer: 0.02 cycles/deg ´ 360 deg/drum =7.2 cycles/drum. Usually, one would fit an even number of cycles inside the drum. The abstract doesn't discuss rotation rate. The reviewer presumes this is constant.

- 'drums evoked equivalent responses with the 0.06 and 0.2 cpd drums'
  Reviewer: What does equivalent mean in this context? Equal?

- 'The saccadic frequency in VA and CS assays was inversely proportional to age, spatial frequency and contrast of the stimulus.'
  Reviewer: Does this mean more saccades at less contrast?

Methods:

- 'From a population of N=50 and N=40 larvae, n=12 larvae were randomly selected for VA and CS experiments at each timepoint for Protocol I and at 16 dpf for Protocol II, respectively. After each experiment, larvae were returned to the tank.'
  Reviewer: Were some larvae reused at several stages in development? It's a major point in animal protocols and best to be clear. The reviewer sees no problem with this as the testing does not cause distress, and if anything, appears to interest the larvae.

- 'CS drums ranging from 100 - 20% were generated by degrading horizontally from the lateral sides to the centre and then changing the transparency percentage of the centre of the black stripes with all retaining 0.02 cycles/degree.'
  Reviewer: This is a difficult sentence.

- 'From another Petri dish, larval or juvenile zebrafish in embryo medium were randomly selected between all larvae in the Petri dish'
  Reviewer: 'From another Petri dish, larval or juvenile zebrafish in embryo medium were

randomly selected'

Results:

- 'saccades per minute were counted'
  Fig. 1. Reviewer: I don't see a camera in the setup. Looks like this is done manually in real time. Sounds like a visual system test for the observer too. Is there at least a clicker?

- Reviewer: In Fig. 1A it is unclear where the second light guide illumination is placed. Is the illumination uniform on the inside of the drum, or is only one hemifield illuminated?

- Reviewer: In Fig. 3, a control with 'no drum' might be useful. One supposes that there might be spontaneous saccades. The fish just 'looks around'. Are the higher spatial frequency responses different from 'no drum'?

- Reviewer: Fig. 4A. It looks like zebrafish are afternoon people, just like the reviewer.

- Legend Fig. 5 'Three replicates of eight larvae, n=24 larvae per pattern.'

- Reviewer: I think that is 24 measurements per pattern.

- Reviewer: In Fig. 4, is it the case among larvae that there are high performers and low performers? There is quite a spread in saccadic eye movements.

Discussion:

- Reviewer comment: The discussion points out that drum evokes responses at higher spatial frequencies and lower contrasts than computerized displays.

- Discussion: 'As zebrafish became older, the saccadic frequency was decreased when spatial frequency was increased.'
  Reviewer: For all ages, saccadic frequency decreased as spatial frequency increased.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My lab has performed OKR research on zebrafish, the topic of the submission.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 18 Dec 2021

Alicia Gómez Sánchez

Dear Dr. Nelson, thank you very much for the constructive review of our work. We really appreciate your suggestions for improvement. In the following paragraphs we address your individual comments. Minor points.
Abstract:

- Reviewer: 0.02 cycles/deg ´ 360 deg/drum =7.2 cycles/drum. Usually, one would fit an even number of cycles inside the drum. The abstract doesn't discuss rotation rate. The reviewer presumes this is constant.

Response: The reviewer is correct and we thank them for pointing this out so that we can clarify. To be more precise we used a 0.0277 cycles/deg. In a 360 deg/drum this equates to 10 cycles/drum. We could not use precisely 0.02, 0.04, 0.06, 0.1 and 0.2 cpd because there would be an incomplete cycle but we designed the patterns as close as possible. We have included this clarification in OKR equipment section in Methods as follows 'The VA drums were 0.0277, 0.0416, 0.0638, 0.1111 and 0.2027 cycles per degree (cpd) were chosen based on a previous publication (15). For convenience, we simply refer to these drums as 0.02, 0.04, 0.06, 0.1 and 0.2 cpd.' Rotation rate is constant at 100 degrees per second so as to not add another variable in the optokinetic response. This parameter is described in Drum Velocity section in Methods 'The drum base was rotated with a constant angular velocity of 100 degrees per second'.

- 'drums evoked equivalent responses with the 0.06 and 0.2 cpd drums'
- Reviewer: What does equivalent mean in this context? Equal?

Response: Please, see Abstract where we have changed 'drums evoked equivalent responses with the 0.06 and 0.2 cpd drums' to '0.06 and 0.2 cpd drums elicited equivalent responses with both set-ups'. They were equivalent because the averages were 7.5 and 5.3 saccades per minute for 0.06 cpd 2D and 3D-printed drum respectively and 7.9 and 5.8 saccades per minute for 0.2 cpd 2D and 3D-printed drum respectively and there was no significant difference between each pair of responses (see Fig. 3)

- 'The saccadic frequency in VA and CS assays was inversely proportional to age, spatial frequency and contrast of the stimulus.'
- Reviewer: Does this mean more saccades at less contrast?

Response: Please see Abstract where we have corrected 'The saccadic frequency in VA and CS assays was inversely proportional to age, spatial frequency and contrast of the stimulus' to 'The saccadic frequency in VA assays was inversely proportional to age and spatial frequency and in CS assays was inversely proportional to age and directly proportional to contrast of the stimulus'. We hope that it is now clarified Methods:

- ‘From a population of N=50 and N=40 larvae, n=12 larvae were randomly selected for VA and CS experiments at each timepoint for Protocol I and at 16 dpf for Protocol II, respectively. After each experiment, larvae were returned to the tank.’
- Reviewer: Were some larvae reused at several stages in development? It’s a major point in animal protocols and best to be clear. The reviewer sees no problem with this as the testing does not cause distress, and if anything, appears to interest the larvae.

Response: The reviewer is correct, some larvae were re-used. In juvenile VA and CS experiments, larvae were returned to the main system tank in compliance with our animal ethics committee approval as the assay does not cause distress and this reduces the number of animals used.

- ‘CS drums ranging from 100 - 20% were generated by degrading horizontally from the lateral sides to the centre and then changing the transparency percentage of the centre of the black stripes with all retaining 0.02 cycles/degree.’
- Reviewer: This is a difficult sentence.

Response: We apologize if our original statement was confusing and have updated the OKR equipment Methods section to ‘CS drums ranging from 100 – 20% were generated from a 0.02 cpd pattern, maintaining the spatial frequency. The black colour of the stripes was changed to include a centre to peripheral vertical gradient generated in Powerpoint™ using the “Gradient Tool, Linear Right Option” and adjusting the transparency of the Mid Gradient Stop according to the desired contrast. For example, 40% black contrast was made by changing the transparency to 60%. (C=100%-60%=40%)’. We hope that is now clear.

- ‘From another Petri dish, larval or juvenile zebrafish in embryo medium were randomly selected between all larvae in the Petri dish’
- Suggested Reviewer Change: ‘From another Petri dish, larval or juvenile zebrafish in embryo medium were randomly selected’

Response: We agree. Please see VA and CS methods (Protocols I and II) Methods section. We have updated our original statement ‘From another Petri dish, larval or juvenile zebrafish in embryo medium were randomly selected between all larvae in the Petri dish’ to ‘From another Petri dish, larval or juvenile zebrafish in embryo medium were randomly selected’ Results:

- ‘saccades per minute were counted’
- Fig. 1. Reviewer: I don’t see a camera in the setup. Looks like this is done manually in real time. Sounds like a visual system test for the observer too. Is there at least a clicker?

Response: We appreciate this comment. We do not routinely use a camera to record the saccades. This was done manually having a timer to count the 30” and record the number of saccades in a lab notebook. Manual counting of saccades is routine and has been published previously (Deeti et al., 2014; Ward et al., 2020; Daly et al 2017 REFERENCE). The OKR activity of 5 dpf wild-type larvae has been completed multiple times, over many years in our lab by different researchers obtaining similar results. Thus, even though it is a manual recording, results are consistent across different observers. Furthermore, we did video-record a subset of the 16 dpf larval OKRs, which were analysed by independent observers, and to address the reviewer’s concern, they validated our results.

- Reviewer: In Fig. 1A it is unclear where the second light guide illumination is placed. Is the illumination uniform on the inside of the drum, or is only one hemifield illuminated?

Response: Reviewer is correct about this observation, the photograph in Fig. 1A does not show clearly where the second light guide is place. We have updated the legend to further explain as follows 'The second light guide is placed on the opposite side to the first guide to uniformly illuminate the inside of the drum'.

- Reviewer: In Fig. 3, a control with 'no drum' might be useful. One supposes that there might be spontaneous saccades. The fish just 'looks around'. Are the higher spatial frequency responses different from 'no drum'?

Response: Previous researchers in our group demonstrated that when the 0.02 cpd drum was not rotated for 30 seconds (in between the clockwise and counter-clockwise rotations) that there very few or no spontaneous saccades. The sum of these were much lower than the average 29.8 saccades per minute we routinely observe in 5 dpf fish with 0.02 cpd 2D/3D-printed drum. In addition, as mentioned to Reviewer 1, we have a mutant zebrafish line with a normal OKR at the 0.02 cpd drum, but a significantly reduced number of saccades compared to siblings at the 0.2 cpd drum. This suggests these movements are predominantly responses to the drum stimuli and not spontaneous saccades when "looking around".

- Legend Fig. 5 'Three replicates of eight larvae, n=24 larvae per pattern.'
- Suggested Change by Reviewer: I think that is 24 measurements per pattern

Response: We appreciate your comment. We have changed in the legend in Fig 5 'Three replicates of eight larvae, n=24 larvae per pattern' to '24 measurements per pattern' which is more accurate in this case.

- Reviewer: In Fig. 4, is it the case among larvae that there are high performers and low performers? There is quite a spread in saccadic eye movements.

Response: We appreciate the reviewer's observation. Eye saccades are an innate response which presents a variability by its biological nature. Even larvae bred together under the same conditions develop at different rates, likely leading to such variability.

Discussion:

- Discussion: 'As zebrafish became older, the saccadic frequency was decreased when spatial frequency was increased.'
- Suggested Reviewer Change: For all ages, saccadic frequency decreased as spatial frequency increased.

Response: We appreciate the reviewer's correction and have corrected our original state 'As zebrafish became older, the saccadic frequency was decreased when spatial frequency was increased' to 'For all ages, saccadic frequency decreased as spatial frequency increased'.

Competing Interests: No competing interests were disclosed

Reviewer Report 12 November 2021

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Logan Ganzen
UC Berkeley, Berkeley, CA, USA

Summary
The authors have presented a method article that describes the development of methods to assess zebrafish vision via the optokinetic response (OKR). Visual acuity (VA) and contrast sensitivity (CS) was assessed by utilizing either a rotating 3D printed striped drum or a 2D paper printed stripe pattern around larval and juvenile zebrafish. By varying stripe spatial frequency, contrast, and chamber illuminance, the authors are able to discern differences in the OKR between conditions. The authors present their method and findings as a low-cost and easy way to reliably assess fine details of zebrafish OKR behavior without needing to purchase expensive computer-assisted technologies. Overall, the article is well-written and scientifically sound. However, there are some addressable concerns with the study.

General Comments

1. One of the justifications provided by the authors for the development of these OKR methods was to potentially "identify more subtle defects in zebrafish vision". While the authors demonstrate that the described methods have the capacity to identify changes in OKR behavior of healthy zebrafish, no direct evidence is provided to demonstrate that the authors' methods are capable of resolving defects in zebrafish vision potentially arising from a disease state. The authors should consider providing an example that one or more of their OKR methods can discern a subtle visual defect in an unhealthy zebrafish as compared to a healthy zebrafish. One potential experiment would be to acutely treat zebrafish larvae with ethanol and demonstrate some of the authors' methods can discern defects in OKR behavior. Previous work has demonstrated that acute EtOH treatment alters the light intensity threshold of zebrafish larvae[1-2]. More recent work has also demonstrated that the $GABA_A$ antagonist picrotoxin can modulate the zebrafish OKR[3]. Pharmacological treatment to alter the OKR could potentially be done quickly and without much difficulty.

2. The authors have gone through the trouble to create a few 3D-printed striped barrels of varying cpd and demonstrated that they are no less effective than their 2D-printed paper barrels in Fig 3. The authors use the "standard 0.02 cpd 3D printed barrel" in Fig. 4 and switch to the 2D barrels for the remaining figures. The authors do not provide much discussion regarding the pros and cons of using either the 3D or 2D barrels. Are the 3D barrels limited in cpd due to the manufacturing process? Is it possible to generate 3D barrels for CS testing? As it stands, the addition of the 3D barrel in the manuscript is somewhat awkward since all of the authors' experiments could have been accomplished with the 2D barrels alone. The authors should critically discuss and justify the use of 3D barrels.

3. The authors have provided the patterns for the 2D barrels in Fig 2. If the manuscript page is printed on a Letter or A4 sheet, are these patterns the proper dimensions to cut and form into a usable barrel? The authors should describe this. For reproducibility of this method between labs to be accurate, the authors should deposit a .svg. .psd, or some other file that contains the pattern on the correct sheet dimension. Additionally, the authors should deposit the files for the 3D-printed barrels as well. The authors could discuss the potentials for the standardization of manual OKR stimuli if these resources are available as open-

source to the community.

Specific Concerns (page # as in pdf)

- Abstract:
  N/A

- Introduction:
  Page 3: “One approach to more thoroughly vision evaluations…” Grammar.
- Page 3: See general comment 2 on 3D barrel justification.

- Methods:
  Page 4: “9% methylcellulose”. Many zebrafish studies publish immobilization in a methylcellulose concentration of around 3%. Would a more viscous solution at 9% potentially alter the saccades per minute statistic measured in the OKR experiments compared with a 3% solution? The authors mention difficulty immobilizing zebrafish beginning at 16 dpf which may call for a higher viscosity solution, but larval fish should sufficiently be immobilized in 3% methylcellulose. The authors should elaborate on this concentration.

- Results:
  Page 9 (and onward): The authors describe values in the text as X number of saccades per minute but do not clearly define if that statistic is an average or a median value of their distribution. Additionally, authors should specify the standard deviation for each statistic they describe.
  Page 9: Figure 3 (and onward): The authors describe the error bars as 1 S.D. but should also explicitly state that the midline of the error bars represents the distribution average (if this is true).
  Page 9/10: Figure 4a. Authors state that the OKR at the 127.5 hpf time point was significantly different from the 121.5 hpf time point on page 9, but Fig. 4a does not reflect this statistical test.
  Page 10: Figure 4a. The light cycle diagram at the top of the figure is probably incorrect. The authors likely meant to indicate that the light turned on at 7:30am, not 7:30pm. The diagram format and alignment are also somewhat confusing.
  Page 10: Figure 4b (and onward). Since the authors have described a time-of-day dependency of the OKR at 5 dpf in Fig 4a, what time of day was the Luminance experiment conducted at, and was the time of day kept consistent for all subsequent experiments and figures? Authors should clarify what time of day the other experiments were performed at.
  Page 10: Figure 5a. The extreme outlier in the 0.1 cpd group may cause confusion with the statistical stars due to its location. Authors may wish to address this.
  Page 12: Figure 6a: The color scheme provided makes it very difficult to discern the colors of 6 dpf and 12 dpf. Authors may wish to consider.
  Page 11/12: Figure 6b. The authors state on page 11 that “In 5 dpf zebrafish, there was no significant difference in OKR using Protocol I or II for 0.2 cpd pattern (Figure 6B)” but do not actually show the data or statistical test for protocol 2 in the figure. Additionally, it appears that the authors have copied the 5 dpf and 16dpf protocol 1 data from Fig. 6a and used it again in Fig. 6b without being explicit that these are the same datasets. This should be made clear to avoid confusion that arises with the statistics being performed (The authors could maintain the color scheme of each protocol 1 dataset from Fig. 6a in Fig. 6b). While the protocol 1 experiment creates paired data which is appropriate for a one-way repeated

ANOVA, protocol 2 creates unpaired data which should not be used with the repeated measures one-way ANOVA described in the figure legend. Comparing protocol 1 and protocol 2 datasets should be done in an unpaired fashion.
Page 13: Figure 7a and b: See previous comments with Figure 6a. Any data used from 7a into 7b should be explicitly stated. Issues with the paired statistical test on unpaired data are present as in 6a and 6b. Should independent protocol 2 data with 100% contrast be added to Fig 7b. for completeness? Fig. 7a appears to be somewhat blurry compared with other figures.

- Discussion:
  Consider elaborating on the 3D barrel as mentioned in general comment 2.

References

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Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of research is generally in visual neuroscience. My doctoral research involved the development of zebrafish behavioral assays to perform drug screening on zebrafish models of disease.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Dec 2021

Alicia Gómez Sánchez

Dear Prof. Ganzen, thank you very much for the constructive review of our work. We really appreciate your suggestions for improvement. In the following paragraphs we address your individual comments. General Comments

1. One of the justifications provided by the authors for the development of these OKR methods was to potentially "identify more subtle defects in zebrafish vision". While the authors demonstrate that the described methods have the capacity to identify changes in OKR behavior of healthy zebrafish, no direct evidence is provided to demonstrate that the authors' methods are capable of resolving defects in zebrafish vision potentially rising from a disease state. The authors should consider providing an example that one or more of their OKR methods can discern a subtle visual defect in an unhealthy zebrafish as compared to a healthy zebrafish. One potential experiment would be to acutely treat zebrafish larvae with ethanol and demonstrate some of the authors' methods can discern defects in OKR behavior. Previous work has demonstrated that acute EtOH treatment alters the light intensity threshold of zebrafish larvae[1-2]. More recent work has also demonstrated that the GABAA antagonist picrotoxin can modulate the zebrafish OKR[3]. Pharmacological treatment to alter the OKR could potentially be done quickly and without much difficulty.

Response: We appreciate this comment. As the reviewer says, we show that the methods to assess VA and CS in zebrafish larvae are effective. Inherently, assays that more sensitively assess vision can uncover more subtle defects in vision. The experiments suggested by the reviewer are indeed interesting, but there is a reasonable chance that these treatments could affect even the standard OKR and therefore would not achieve the objective. However, we have assessed VA and CS in two zebrafish knockout lines. In one case, there was no change compared to siblings. In another, a significant VA/CS defect was observed only in the mutant compared to siblings with the highest spatial frequency and lowest contrast sensitivity. These pieces of data are being included in other manuscripts (one under review) wherein those genes are the focus of the manuscripts. Therefore, it is best to include that VA/CS data in those manuscripts. In summary, the assays will not always uncover more subtle defects in vision, but fundamentally they can because they require better visual acuity or contrast sensitivity. We have an example in a mutant line and this will be published shortly corroborating these methods. However, the main findings from this manuscript strand alone.

1. The authors have gone through the trouble to create a few 3D-printed striped barrels of varying cpd and demonstrated that they are no less effective than their 2D-printed paper barrels in Fig 3. The authors use the "standard 0.02 cpd 3D printed barrel" in Fig. 4 and switch to the 2D barrels for the remaining figures. The authors do not provide much discussion regarding the pros and cons of using either the 3D or 2D barrels. Are the 3D barrels limited in cpd due to the manufacturing process? Is it possible to generate 3D barrels for CS testing? As it stands, the addition of the 3D

barrel in the manuscript is somewhat awkward since all of the authors' experiments could have been accomplished with the 2D barrels alone. The authors should critically discuss and justify the use of 3D barrels.

Response: Thank you for your question. Here, we present two affordable set-ups to measure visual behaviour in zebrafish. 2D drums have the advantage of being printed inexpensively, however, they are printed on cardboard which can be broken or deformed easily. 3D drums, by contrast, are made of PLA, which makes the drum rigid, harder to break and thus, longer lasting. Nevertheless, an external company manufactures the 3D drums, so the cost price is higher (less than 100€/drum) than 2D drums but being still considerably more affordable than an automated OKR. We started with the2D-printed drums as they could quickly and affordably be generated in-house. For sustainable, long-term use of the same drums we consider the 3D drums a better option and therefore we considered it important to prove that both set-ups work in practice and yield equivalent results which we show with the VA drums. We have clarified this point at the end of the introduction as follows 'Here, we describe two simple and affordable methods to assess VA and CS in zebrafish using either 2D and 3D printed striped patterns/drums to quantify OKR, VA and CS in larval and juvenile zebrafish'. We also discuss this point in the Discussion section with the heading Affordability and Accessibility which has been updated to state: 'While 2D-printed can be easily printed on cardboard, they are less durable so can be used for pilot experiments. However, if researchers prefer a more durable option, 3D-printed drums are still an affordable option (less than 100€ (per drum) yielding equivalent results as 2D-printed drums'

1. The authors have provided the patterns for the 2D barrels in Fig 2. If the manuscript page is printed on a Letter or A4 sheet, are these patterns the proper dimensions to cut and form into a usable barrel? The authors should describe this. For reproducibility of this method between labs to be accurate, the authors should deposit a .svg. .psd, or some other file that contains the pattern on the correct sheet dimension. Additionally, the authors should deposit the files for the 3D-printed barrels as well. The authors could discuss the potentials for the standardization of manual OKR stimuli if these resources are available as open-source to the community.

Response: We appreciate your suggestions. Patterns in Fig 2 are ready to cut and build if printed on A4 sheets. For reproducibility purposes, please see OKR equipment Methods section where we provide company contact details (projects@materialise.co.uk) and all details necessary (drum dimensions: 3.4 cm high and 6 cm in diameter; desired cycles per degree: 0.02, 0.04, 0.06, 0.1 or 0.2 cpd and stripes colour: black and white) to get 3D drums manufactured as ours. The files to print the 3D drums were generated by the commercial supplier and we do not have access to them to deposit them as queried. We agree that standardization of OKR stimuli (indeed the overall assay) is a worthy goal and therefore we have included all the details of the patterns that we have in the Methods and have considered several causes of variability in OKR assays in the Discussion. Specific Concerns (page # as in pdf)

- Introduction: Page 3: "One approach to more thoroughly vision evaluations…" Grammar.

Response: We agree and have changed our original statement: 'One approach to more thoroughly vision evaluations…' to 'One approach to assessing vision more thoroughly is to vary the optokinetic stimulation'.

- Page 3: See general comment 2 on 3D barrel justification.

Response: This point was also addressed above. We have updated the Affordability and Accessibility section in the Discussion. We have discussed the differences between 2D and 3D-printed drums as follows 'While 2D-printed can be easily printed on cardboard, they are less durable so can be used for pilot experiments. However, if researchers prefer a more durable option, 3D-printed drums are still an affordable option (less than 100€ (per drum) yielding equivalent results as 2D-printed drums'

- Methods: Page 4: "9% methylcellulose". Many zebrafish studies publish immobilization in a methylcellulose concentration of around 3%. Would a more viscous solution at 9% potentially alter the saccades per minute statistic measured in the OKR experiments compared with a 3% solution? The authors mention difficulty immobilizing zebrafish beginning at 16 dpf which may call for a higher viscosity solution, but larval fish should sufficiently be immobilized in 3% methylcellulose. The authors should elaborate on this concentration.

Response: We appreciate this comment and the need for researchers to elaborate on the methylcellulose used in their studies. Previous studies have also used more than 3% methylcellulose (i.e. 6% or 9%) (e.g. Brockerhoff et al, 20061; Zou et al, 20102; Deeti et al 20143; Daly et al, 20174; Ward et al, 20205; Ward et al, 20206) (see references at the end) obtaining a similar number of saccades at 5 dpf as we report here. Notably, there are different grades of methylcellulose with different viscosities (cP values). We routinely used methylcellulose of 25 cP (Sigma M6385) in our studies. We do not have ethical approval to place zebrafish animals older than 131 hpf in methylcellulose >9%.

Results:
Page 9 (and onward): The authors describe values in the text as X number of saccades per minute but do not clearly define if that statistic is an average or a median value of their distribution. Additionally, authors should specify the standard deviation for each statistic they describe. Response: We have clarified it in Statistical analysis methods section as follows 'All saccades per minute values described in this manuscript are a group average'. The standard deviation has been included for each statistic value (saccades per minute group average) described in the manuscript text. Page 9: Figure 3 (and onward): The authors describe the error bars as 1 S.D. but should also explicitly state that the midline of the error bars represents the distribution average (if this is true). Response: We apologise for this oversight and included a statement in the legend of Figure 3 and onward as follows 'Midline of error bars represents the group average' Page 9/10: Figure 4a. Authors state that the OKR at the 127.5 hpf time point was significantly different from the 121.5 hpf time point on page 9, but Fig. 4a does not reflect this statistical test. Response: Figure 4a has been updated and now reflects the statistical difference between 121.5 and 127.5 hpf.
Page 10: Figure 4a. The light cycle diagram at the top of the figure is probably incorrect. The authors likely meant to indicate that the light turned on at 7:30am, not 7:30pm. The diagram format and alignment are also somewhat confusing. Response: We thank the reviewer for spotting this error. The light cycle diagram has been modified for better understanding. We hope it is now clearer.
Page 10: Figure 4b (and onward). Since the authors have described a time-of-day dependency of the OKR at 5 dpf in Fig 4a, what time of day was the Luminance experiment conducted at, and was the time of day kept consistent for all subsequent experiments and

figures? Authors should clarify what time of day the other experiments were performed at. Response: We appreciate this comment and have included what time of day the experiments from Fig 4b and onwards were performed. Experiment from Fig. 4b was done at 125 hpf (please see updated The zebrafish larval OKR response is modulated by time of day and luminance levels in Results section (Pag. 11)) Experiment from Fig. 5a was done at 123 hpf which was included in the original manuscript (please see 'The 2D/3D-printed striped patterns enable discrimination of VA and CS in larval zebrafish' in Results section (Pag. 11)) Experiment from Fig. 5b was done at 125 hpf which was included in the original manuscript (please see 'The 2D/3D-printed striped patterns enable discrimination of VA and CS in larval zebrafish' in Results section (Pag. 11)) Experiment from Fig. 6a was done at 123 hpf (please see updated 'The zebrafish VA response shows age-dependent variations' in Results section (Pag. 11)) Experiment from Fig. 6b was done at 123 hpf (please see updated 'The zebrafish VA responses show age-dependent variations' in Results section (Pag. 11 and 14)). Experiment from Fig. 7a was done at 125 hpf (please see updated 'The zebrafish CS response shows age-dependent variations' in Results section (Pag. 11)) Experiment from Fig. 7b was done at 125 hpf (please see updated 'The zebrafish CS responses show age-dependent variations' in Results section (Pag. 11 and 14))
Page 10: Figure 5a. The extreme outlier in the 0.1 cpd group may cause confusion with the statistical stars due to its location. Authors may wish to address this. Response: We agree and have updated Figure 5a accordingly.
Page 12: Figure 6a: The color scheme provided makes it very difficult to discern the colors of 6 dpf and 12 dpf. Authors may wish to consider. Response: We have changed the color scheme for all groups in Figure 6 and 7 which it is now more visual.
Page 11/12: Figure 6b. The authors state on page 11 that "In 5 dpf zebrafish, there was no significant difference in OKR using Protocol I or II for 0.2 cpd pattern (Figure 6B)" but do not actually show the data or statistical test for protocol 2 in the figure. Additionally, it appears that the authors have copied the 5 dpf and 16dpf protocol 1 data from Fig. 6a and used it again in Fig. 6b without being explicit that these are the same datasets. This should be made clear to avoid confusion that arises with the statistics being performed (The authors could maintain the color scheme of each protocol 1 dataset from Fig. 6a in Fig. 6b). While the protocol 1 experiment creates paired data which is appropriate for a one-way repeated ANOVA, protocol 2 creates unpaired data which should not be used with the repeated measures one-way ANOVA described in the figure legend. Comparing protocol 1 and protocol 2 datasets should be done in an unpaired fashion. Response: We wish to clarify that the bottom right corner of the Fig 6B graph for 5 dpf larvae shows there is no significant difference for the 0.2 drum between the protocols. We thank the reviewer for the opportunity to clarify this point. We do re-use the from 5 and 16 dpf Protocol I datasets from Fig 6a in Fig 6B (which include new data for Protocol II) as conditions were the same and we have a limited number of approved fish for these experiments under our ethical approval. We have clarified this in the revised manuscript updating The zebrafish VA responses shows age-dependent variations Results section (Pag. 11) as follows 'In Figure 6b, datasets from 5 and 16 dpf groups for Protocol I are from the same groups in Figure 6a'. We thank the reviewer for spotting this incorrect sentence in the legend. The data from Figure 6B was analysed using one-way ANOVA unpaired measures and Tukey's multiple comparisons test and this has been revised in the Statistical Analysis methods section and in the figure legend. Page 13: Figure 7a and b: See previous comments with Figure 6a. Any data used from 7a into 7b should be explicitly stated. Issues with the paired statistical test

on unpaired data are present as in 6a and 6b. Should independent protocol 2 data with 100% contrast be added to Fig 7b. for completeness? Fig. 7a appears to be somewhat blurry compared with other figures. Response: We have clarified this, updating The zebrafish CS responses shows age-dependent variations Results section (Page. 11/Page.14) as follows 'In Figure 7b, datasets from 5 and 16 dpf groups for Protocol I are from the same groups in Figure 7a' (Page 14). We thank the reviewer for spotting this incorrect sentence in the legend. The data from Figure 7B was analysed using one-way ANOVA unpaired measures and Tukey's multiple comparisons test and this has been revised in the Statistical Analysis methods section and in the figure legend. Protocol II refers to when the larvae are naïve to a stimulus whereas in Protocol I the larvae move form highest contrast (100%) to lower contrast stimuli. Therefore, the 100% contrast is actually the naïve response in Protocol II. The resolution of Fig 7a may have been reduced in the process of generating a PDF. We have revised the figure to be of higher-resolution. Discussion:
Consider elaborating on the 3D barrel as mentioned in general comment 2. Response: This was addressed earlier and is revised in the Discussion section.

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Competing Interests: No competing interests were disclosed.