

Contrast Sensitivity, Spatial and Temporal Tuning of the Larval Zebrafish Optokinetic Response

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PURPOSE. To characterize the quantitative properties of the optokinetic response (OKR) in zebrafish larvae as a tool to test visual performance in genetically modified larvae.

METHODS. Horizontal OKR was triggered in 5-day-old zebrafish larvae by stimulation with projected computer-generated gratings of varying contrast, angular velocity, temporal and spatial frequency, and brightness. Eye movements were analyzed by a custom-made eye tracker based on image analysis.

RESULTS. The gain of the OKR slow phase was dependent on angular velocity, spatial frequency, and contrast of a moving grating, but largely independent on brightness. Eye velocity was a logarithmically linear function of grating contrast with a slope of approximately 0.8 per log unit contrast.

CONCLUSIONS. The OKR of the larval zebrafish is not scaled for stimulus contrast and spatial frequency. These properties make the OKR a valuable tool to quantify behavioral visual performance such as visual acuity, contrast sensitivity, and light adaptation. This behavioral paradigm will be useful for analyzing visual performance in mutant and gene-knockdown larval zebrafish. (*Invest Ophthalmol Vis Sci.* 2005;46:137-142) DOI: 10.1167/iovs.04-0682

The zebrafish (*Danio rerio*) has become an established model system for genetic studies of vertebrate vision. The zebrafish visual system develops extraordinarily rapidly. As soon as 5 days postfertilization (dpf), the visual system is well developed by morphologic, electrophysiological, and behavioral criteria (reviewed in Bilotta¹). It supports a number of visually mediated behaviors that have been used to identify mutant strains with defects in vision (reviewed in Neuhauss²). Such behaviors are mainly based on the detection of motion cues, resulting in stereotypic eye movements (optokinetic response) in larvae or in directed swimming movements (optomotor response) in larvae³ and adults.⁴⁻⁶

Genetic screens for zebrafish strains defective in vision, based on such visual behavioral responses, led to the isolation of a number of mutant strains of interest.^{4,7-14} Simple screens of visual behavior in mutant strains may not be sensitive

enough to detect subtle defects in the visual system. Hence, a thorough analysis of visual performance in zebrafish necessitates robust behavioral paradigms able to uncover subtle alterations in visual performance. In adult zebrafish, Maaswinkel and Li⁶ showed that visual performance can be characterized based on the optomotor response. For larval zebrafish we developed a system to measure visual performance quantitatively under various experimental conditions based on the optokinetic response (OKR).

The OKR has been used as a behavioral assay to explore the contrast sensitivity function in cats¹⁵ and humans.^{16,17} This behavior does not need training and it becomes functional after 3 dpf in zebrafish larvae.¹⁸⁻²⁰ The optomotor system is a closed loop,²¹ which adjusts eye velocity according to a retinal slip signal, the difference between pattern movement and eye velocity. At the beginning of this loop are motion sensors. These motion sensors have been thoroughly studied in insects.²²⁻²⁴ Similar models derived from these invertebrate studies have been proposed to be valid for human motion perception as well.²⁵ In all models these motion sensors do not code for pure velocity but depend on textural properties such as temporal and spatial frequency and contrast. In vertebrates, the visual system can partially compensate for texture. De Graaf et al.²⁶ showed that velocity rather than temporal or spatial frequency determines velocity perception as well as the optokinetic response. Furthermore there is only a minor influence of pattern contrast on motion perception.²⁷ The optokinetic response, however, is contrast dependent in humans¹⁷ and cats.¹⁵ The goal of this study was to analyze the optokinetic response in wild-type larval zebrafish to establish psychophysical paradigms for assaying visual performance. To this end, we determined the optokinetic gain as a function of contrast, angular velocity, spatial and temporal frequency, and brightness.

METHODS

Fish Maintenance and Breeding

Wild-type fish from the inbred WIK strain were bred and crossed as previously described.²⁸ Embryos were raised at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) and staged according to development in days postfertilization (dpf). If not otherwise stated, the WIK wild-type strain was used throughout the study. Similar results have been observed by using the TU wild-type strain.

Optokinetic Stimulation

At 5 dpf, larvae were randomly chosen from a single clutch. All measurements were done in the afternoon between 2 PM and 6 PM.

For full-field stimulation, single larvae were placed dorsal up in the center of a Petridish (35 mm diameter) containing 3% prewarmed (28°C) methylcellulose to suppress whole-body movement without constricting eye movements. Moving gratings were projected by a digital light projector (DLP; Proxima 4200; Proxima, Wilsonville, OR) onto a screen within the visual field of the larva, at an apparent distance of 4.65 cm from the larva's eye. DLP projectors are suited for psychophysical experiments because they can be well characterized in terms of timing and input-output relationship.²⁹ Projection was fo-

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cused on a cylindrical diffusion screen by combining a 50 mm lens with a 100 mm lens. Projection size on the screen was 99° horizontally and 52° vertically. A SVGA graphics board (NVIDIA GeForce 4, Santa Clara, CA) with a frame rate of 60 Hz and 8-bit intensity resolution controlled by Microsoft Windows XP (Redmond, WA) generated the stimulation patterns. A custom-made graphics library based on the *Simple DirectMedia Layer* (SDL, <http://www.libsdl.org>), allowing full control of timing and intensity of the projection was used to create the stimuli. The image was wrapped on a cylindrical screen by a mapping function defined by:

$$x' = R \cdot \tan\left(\frac{x}{R}\right)$$

where x' is the horizontal coordinate on the screen, x the position where the ray would hit a flat screen, and R the radius of the screen. The input-output relation of the projector was linearized by hardware reset of the lookup tables, making use of the intrinsic linearity of the DLP technique. Linearity was confirmed photometrically. Effective intensity at the position of the larva was measured by a photometer (Tektronics J18; Beaverton, OR). Accordingly the contrast C of grating stimuli was calculated from the intensity of the sine wave maximum (I_{\max}) and minimum (I_{\min}) as:

$$C = (I_{\max} - I_{\min}) / (I_{\max} + I_{\min})$$

The minimal and maximal contrast that could be achieved with the projector was 0.7% and 99.0%, respectively. Mean luminance levels were adjusted by introducing neutral density filters into the light beam. The maximal intensity was 5230 cd/m².

Recording of Eye Movements

Bright field image sequences of the larval head were observed using a custom modified infrared black/white video camera (Sony XT-SC50; Berlin, Germany) attached to a binocular microscope with a phototube (SV8; Zeiss, Oberkochen, Germany). Larvae were illuminated from below with infrared light to avoid interference with the light stimulus. The camera was customized with an electronic circuit for on-chip frame integration recording with up to 12.5 frames/s. If not otherwise indicated, eye movements were tracked at 5 frames/s throughout the study. For digitization of the video signal, a programmable video grabber card (PCI-1409; National Instruments, Austin, TX) was used. The camera's recording speed was synchronized by dedicated electronic measures.

Custom-developed software on the basis of LabView IMAQ (version 5.1; National Instruments) was used to control stimulation and camera, and to analyze the resulting images. The software extracts the shape of the eye from the image by applying a series of image processing algorithms to the image based on the darker pigmentation of the eye. After adaptive contrast correction and smoothing employing a convolution filter, an adaptive threshold prepares the image for particle separation by using a combination of erosion and dilation. After recognition of the ellipse-like shape of the eye with a particle filter method, particle analysis algorithms were applied to identify the eye's maximum intercept and its orientation in relation to the horizontal axis. In addition, the center coordinates of both eyes were tracked to correct small movements of the larvae over time, considering horizontal movements as well as rotation. Both image recording and analysis were achieved in real time and were monitored during the experiment on a computer screen.

Experimental Procedure

Constrained larvae were stimulated monocularly with sinusoidal gratings. The right eye was stimulated, while the left eye field consisted of a dark, low-contrast surface. Direction of pattern movement was varied by a temporal square wave function of 0.17 Hz. Eye movements, eye

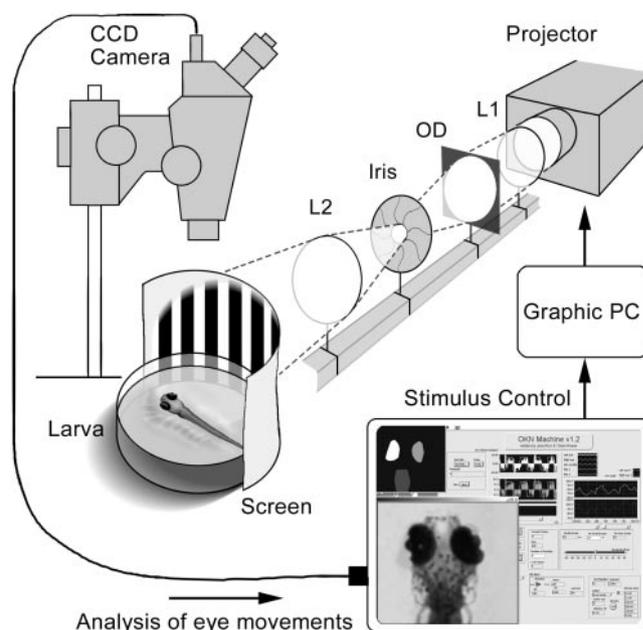


FIGURE 1. Schematic drawing of the setup used to stimulate and record optokinetic responses (not drawn to scale). Immobilized zebrafish larvae are stimulated with gratings projected onto a cylindrical screen. Projection is focused through two convex lenses (L1, L2). Intensity is adjusted by optical density filters (OD). Eye movements are recorded with a CCD camera and evaluated in real-time in terms of eye angle and velocity.

velocity, and image configuration were recorded automatically. Variation of grating variables such as contrast occurred according to a schedule in a single trial. Averaged eye velocity for each experimental condition was calculated by integration of eye velocity. Only the slow phase of the optokinetic response was recorded, because the fast saccadic movements occurred with a fixed velocity. Consequently, before the integration step, the velocity recordings were filtered for saccades. If the eye velocity (v) in a frame (f) exceeded a certain threshold indicative of a saccade (threshold was set to gain > 3), $v(f \dots f - 2)$ was set to $v(f - 3)$, $v(f = +1 \dots f + 2)$ was set to $v(f + 3)$. With this procedure, eye velocity recordings were smoothed around the spikes caused by saccades. Additionally $v(f)$ was averaged with a sliding window of three frames.

RESULTS

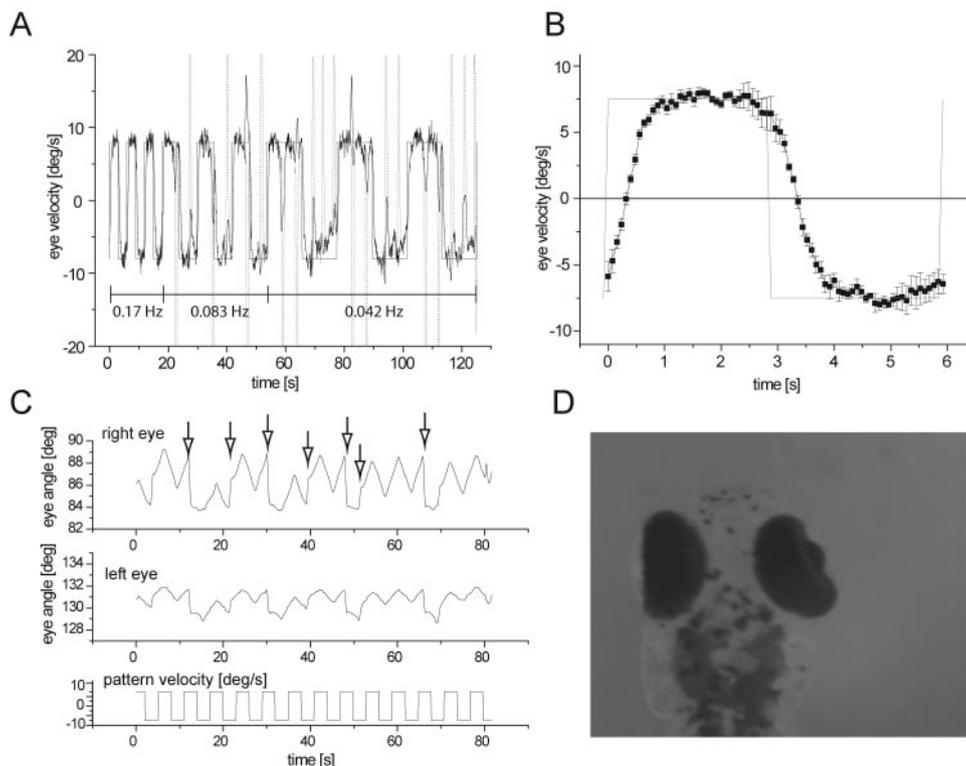
Precise Measurement of Larval Eye Movements by Image Analysis

In our experimental set-up, an immobilized 5-day-old larva was placed in the center of a plastic dish, viewing a screen. Motion stimuli were presented by projecting computer-generated patterns with a video projector onto the screen, viewed by the right eye of the larva. Eye movements were recorded by a video camera through a dissecting scope, and analyzed in real time with custom-made software (see Fig. 1 and method section for details). Recently Roeser et al.³⁰ described a procedure also based on image analysis of eye movement that was used to study the role of the optical tectum in optomotor control.

The OKR can be reliably evoked in 5-day-old larvae, and a number of mutant strains with visual defects have been identified at this stage. Therefore, most mutants are assayed at this stage for visual defects.

The most direct approach to devise psychophysical methods for evaluation of visual performance is to determine thresholds of detection. For example, the contrast at which optoki-

FIGURE 2. OKR measured with moving gratings of alternating direction. Stimulus conditions in all panels are: contrast = 99%, $v = 7.5^\circ/\text{s}$, $sf = 0.06$ cycles/degree, brightness = 41 cd/m^2 . **(A)** OKR measured under varying frequencies of movement direction alternation (0.17 Hz, 0.083 Hz, 0.042 Hz). The unfiltered recording containing the saccades is indicated with a dotted line. With longer duration of unidirectional stimulation the number of saccades increases because the maximal possible eye amplitude is exceeded. Also there is a slight decrease in gain with longer unidirectional stimulation. **(B)** OKR buildup with alternating movement direction. Saccade-free recordings of $n = 8$ larvae were averaged after approximately 20 s prestimulation. OKR buildup is largely complete after 1 s. **(C)** Optokinetic response from a single animal stimulated with a grating alternating in movement direction with 0.17 Hz (lowest panel). Arrows, saccades between smooth tracking movements. Only the right eye is stimulated. The left eye follows the movements of the right eye with reduced velocity. **(D)** Sample frame of Movie 1 (see www.iovs.org/cgi/content/full/46/1/137/DC1) which shows 20 s of the recording from which Figure 2C was derived. The larva is embedded dorsally up and illuminated from below with infrared light. The right eye faces a screen where stimuli are projected.



netic eye movements can no longer be evoked could be measured. Such approaches using optokinetic eye movements have been successfully performed in humans.¹⁶ The drawback of this method is the criterion for the absence of an optokinetic response. Conversely, it can be judged by visual inspection of the recordings by applying some arbitrary threshold for a minimum eye velocity accepted as optokinetic response. The first approach poses more challenges for automated processing of eye movements. The second approach suffers from artifacts caused by spontaneous eye angle drifts, which are difficult to discriminate from slow optokinetic movements.

To circumvent these problems, we applied another experimental paradigm, using the gain (ratio of eye velocity and stimulus velocity) of the OKR slow phase as an objective way to measure contrast sensitivity. In cats and humans, the gain of the OKR elicited by a moving grating of constant velocity is a function of stimulus properties like angular velocity and contrast.^{15,17} Assuming that this relationship also holds true for zebrafish larvae, a robust psychophysical function can be obtained by measuring the gain in relationship to any stimulus variable. Such a psychophysical function would circumvent the problem of evaluating weak optokinetic responses at the visual threshold. We noticed that presentation of unidirectional motion stimuli resulted in a decrease in gain, which made reproducible measurements difficult (Fig. 2A). However, alternating the direction of the motion stimulus with a frequency of 0.17 Hz abolished this effect. This frequency was chosen after testing the time needed for buildup of the OKR. Another effect of this alternating movement was the reduction in the number of saccades that occur (Fig. 2A), because eye movements can occur within each phase without exceeding the maximal amplitude. This reduces noise caused by imperfect filtering of saccades. Roeser et al.³⁰ showed that ablating the optic tectum reduces saccade frequency with only slight reduction of OKR gain. Dedicated saccade-generating circuits

in the hindbrain are responsible for saccade timing.³¹ Consequently the OKR slow phase can be examined without taking saccades into account.

When the larva is stimulated with a moving grating (contrast = 99%, $v = 7.5^\circ/\text{s}$, $sf = 0.06$ cycles/degree, recording frame rate 12.5 frames/s) the maximal gain is reached after approximately 1 second (Fig. 2B). Therefore, a 3-second stimulation with a grating moving in one direction is sufficient to reach the maximal gain. In our setup, only the right eye was stimulated. The left eye field consisted of a dark, low-contrast surface. Although the left eye received no motion stimulus, eye movements were yoked. The unstimulated eye followed the stimulated eye albeit with reduced gain (Fig. 2C). Movie 1 (see www.iovs.org/cgi/content/full/46/1/137/DC1) shows the recording from which Figure 2C was derived.

Optokinetic Gain as Function of Stimulus Velocity, Spatial and Temporal Frequency

Next, we examined which stimulus properties influence the gain of the OKR. The optokinetic system could be sensitive to temporal frequency or could be scaled to angular velocity of the moving grating. To distinguish between these alternatives, spatial frequency and velocity of a grating stimulus were varied. Because temporal frequency (TF) is related to spatial frequency (SF) and angular velocity (V) by $TF = V \cdot SF$, all three parameters of interest were varied in this experiment.

Larvae were stimulated with gratings varying in spatial frequency with a fixed pattern contrast of 79%. In the first epoch, angular velocity was set to $3.75^\circ/\text{s}$. Then the same set of spatial frequencies was presented with $7.5^\circ/\text{s}$, $15^\circ/\text{s}$, and $22.5^\circ/\text{s}$, respectively.

Figure 3 shows the results from this experiment with $n = 6$ larvae. The slow phase of the OKR was markedly different for a given temporal frequency with varying angular velocity, es-

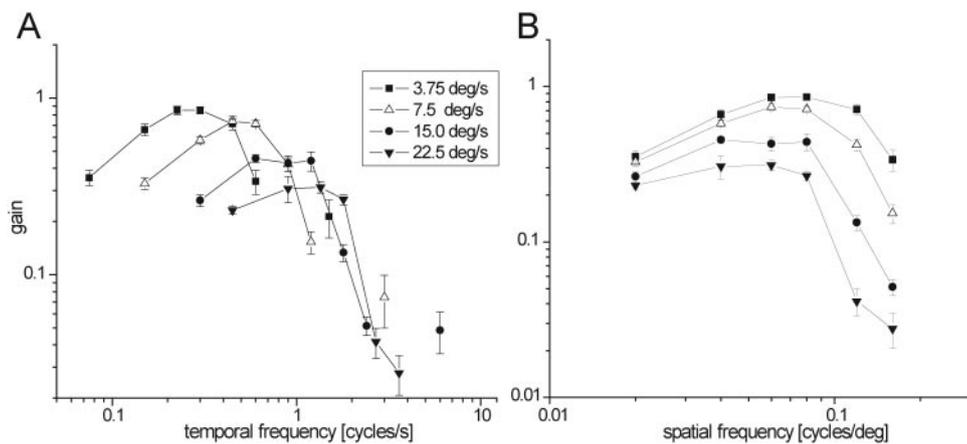


FIGURE 3. OKR slow phase depends on temporal and spatial frequency of a moving sine grating. Graphs show averaged data from $n = 5$ larvae. Contrast was fixed at 79%. **(A)** Eye velocity as function of temporal frequency for different drum velocities. **(B)** Eye velocity as function of spatial frequency. Eye velocity shows a strong dependence on spatial frequency. Error bars, SEM.

pecially for higher spatial frequencies (Fig. 3A). When the spatial frequency was the independent variable, the normalized curves overlapped (Fig. 3B). At higher angular velocity, the gain was markedly reduced for all spatial frequencies, presumably due to the incapability of the OKR to follow at higher velocities. There was also an interaction between spatial frequency and angular velocity (two-way ANOVA for repeated measurements [$F(df = 15) = 16.9, P < 0.001$]) that reflected a reduction in gain at higher spatial frequencies under high angular velocity stimulation.

Independent of the temporal aspects, there was a strong dependence of the gain on the spatial frequency of the moving grating. The SF-gain function showed a maximum at approximately 0.06 cycles/degree.

Optokinetic Gain-Dependence on Contrast

To test the contrast dependence of the OKR slow phase in zebrafish, larvae were stimulated with a sine grating of constant spatial frequency of 0.06 cycles/degree and an angular velocity of 7.5°/s in a similar manner as in the previous experiment but with contrasts varying between 5% and 99%. The averaged results ($n = 6$) are shown in Figure 4A. The contrast-gain function has a remarkably shallow slope of 0.76 per log unit. The gain is a linear function of the logarithm of contrast and saturates only at very high contrast levels. To test if this linear relationship can be generalized to varying spatial frequencies, we measured contrast-gain curves for various spatial

frequencies. For all spatial frequencies tested, the OKR slow phase velocity was a linear function of logarithmic contrast (Fig. 4b). We calculated contrast sensitivity as a function of spatial frequency by linear regression of the contrast gain curves. The results are given in Table 1. To examine this relationship in the larval development, we measured contrast-gain curves in 4, 5, and 6 dpf larvae. Data were collected from the same clutch of an inbred TU strain. The contrast-gain relationship shown in Table 1 is valid for all developmental stages measured.

Taken together, these experiments showed that the optokinetic gain in larval zebrafish is not scaled for stimulus contrast. Over a broad range of grating contrasts, optokinetic gain increased linearly with the logarithm of contrast.

Brightness Dependence of the Optokinetic Gain

In the previous experiments, we characterized the optokinetic response as a function of spatial, temporal, and contrast properties of stimulating gratings. We found that the reaction strength measured as optokinetic gain for a broad range of spatial frequencies and angular velocities was not scaled for contrast. Another important perceptual constancy in vertebrates is the ability to adapt to changes in light intensity. Over a broad range of intensities, the vertebrate eye can shift its working range to accommodate to an impressive range of ambient light levels. Therefore, we explored the slow phase of the optokinetic response under varying mean intensities of the

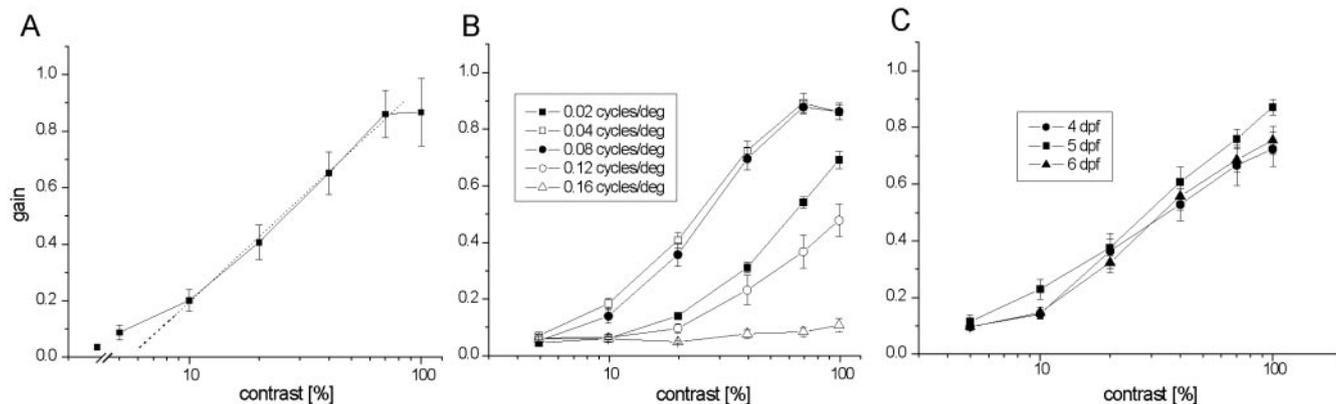


FIGURE 4. OKR slow phase as function of contrast indicates absence of an effective contrast-gain control in the optokinetic response. Larvae were stimulated with moving gratings ($v = 7.5^\circ/s, sf = 0.06$ cycles/degree, brightness = 41 cd/m²). Contrast was varied between 4.9% and 99%. **(A)** Averaged ($n = 12$) contrast-gain function shows a shallow slope (0.77 per log unit) which is over a wide range of grating contrasts linearly related to the logarithm of contrast. **(B)** The contrast-gain relationship is also logarithmically linear under a broad range of spatial frequencies. **(C)** Larvae from a single clutch of an inbred TU wild-type strain were measured at different developmental stages (4 dpf, 5 dpf, 6 dpf).

stimulating grating. Contrast-gain curves were measured under different mean light intensities. Intensity was adjusted by neutral density filters. Because the focused image of the DLP projector is very bright (maximal 5230 cd/m² at larva's position), image intensity can be varied over several orders of magnitude. Larval zebrafish ($n = 6$) were stimulated with gratings of constant spatial frequency of 0.06 cycles/degree. Contrast was varied between 1% and 99%.

Contrast sensitivity varies as a function of image intensity with maximum sensitivity at 41 cd/m² average intensity (Fig. 5). The optokinetic response adapts to large differences in brightness levels. Above brightness levels of approximately 3 cd/m², contrast sensitivity remained constant.

DISCUSSION

A function of the optokinetic response is to stabilize the visual world on the retina. Accordingly, in larval zebrafish, as in cats¹⁵ and humans,²⁶ the optokinetic response is a function of angular velocity rather than temporal frequency. However, the optokinetic gain is also dependent on spatial frequency of the stimulating pattern. Above an intensity of approximately 3 cd/m², optokinetic gain is almost independent of brightness. Mutants with specific adaptation defects such as light adaptation would be expected to show a decrease in gain for high intensities. Besides this there is apparently no effective contrast gain control for the optokinetic response. Optokinetic gain increases linearly with the logarithm of pattern contrast for all spatial frequencies measured. It is unclear why there is an absence of contrast-gain control. There is good evidence that the optokinetic response in teleosts is not markedly controlled by the optic tectum.³² Ablation studies in adult goldfish³² and larval zebrafish³⁰ show that the OKR is only marginally compromised by optic tectum ablation. One can speculate that this low-level circuit is not scaled for stimulus properties such as contrast and spatial frequency. At a later processing state, the optic tectum could do the necessary scaling to perceive the angular velocity of moving objects independently of their texture in a biological, meaningful manner. In flies it has been shown that activity of HS-cells which perform spatial integration of movement information in the lobula are contrast dependent. Also the optomotor behavior of flies shows some contrast dependence.²²

Because the optokinetic response can be measured swiftly and precisely with our procedure, the method is suitable to characterize mutant larvae efficiently. Moreover, the OKR can be reliably measured as early as 4 dpf. Thus, this method will also be useful in characterizing larvae treated with morpholino antisense oligonucleotides where the knockdown effect ceases over time. For such purposes, the apparent absence of contrast gain-control is an advantage. Instead of constructing psychometric functions by threshold measurements, the contrast-gain relationship is inherently a psychometric function by itself. The dependence on spatial frequency is advantageous to assay

TABLE 1. Estimation of Contrast Sensitivity as Function of Spatial Frequency

Spatial Frequency (cycles/deg)	A	B	Contrast Sensitivity
0.02	-0.84	0.75	7.57
0.04	-0.67	0.85	16.39
0.08	-0.75	0.88	14.29
0.12	-0.58	0.52	7.69
0.16	n.d	n.d.	n.d.

$$\text{Optokinetic gain} = A + B \cdot \log(\text{Contrast}).$$

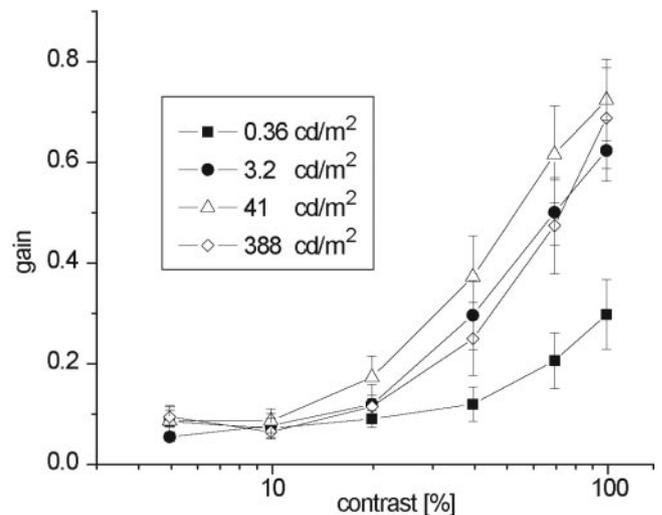


FIGURE 5. Contrast sensitivity varies only slightly with large differences in mean intensity. OKR slow phase contrast sensitivity under various mean grating intensities ($v = 7.5^\circ/s$, $sf = 0.06$ cycles/degree). Contrast was varied between 4.9% and 99% with mean grating intensities between 0.36 cd/m² and 388 cd/m². This indicates functional light adaptation in larval zebrafish.

spatial vision in larval zebrafish. By measuring the gain as a function of spatial frequency, a relationship is obtained which is comparable to a contrast sensitivity function (CSF) because the gain is a function of contrast and spatial frequency. It is noteworthy that this OKR-CSF is not based on threshold measurements but rather uses the contrast dependence of the OKR-gain as an indirect measure of contrast sensitivity. The CSF in humans is very sensitive to even subtle defects in vision. This property is used in the clinic and a large number of ophthalmic and neurologic conditions have been shown to affect the CSF.³³⁻³⁸ A cataract, for instance, causes an overall blurring of the image, thereby decreasing the CSF amplitude over the whole range of spatial frequencies. Conditions affecting the receptor arrangement or spacing will affect mostly high spatial frequencies. Accordingly, the OKR-CSF can be used as a sensitive assay to evaluate zebrafish mutants and morpholino knockdown larvae with potential ophthalmic and neurologic deficits.

In summary, we have explored properties of the OKR in larval zebrafish, demonstrating that the gain of this behavior depends on stimulus velocity and spatial frequency, but only slightly on brightness. Furthermore, optokinetic gain is dependent on stimulus velocity rather than on temporal frequency. These properties will be useful to quantify visual performance in genetically modified zebrafish larvae.

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