Chapter 10

Analysis of Optokinetic Response in Zebrafish by Computer-Based Eye Tracking

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Abstract

Large-field movements in the visual surround trigger spontaneous, compensatory eye movements known as optokinetic response (OKR) in all vertebrates. In zebrafish (*Danio rerio*) the OKR is well developed at 5 days post fertilization and can be used in the laboratory for screening of visual performance following genetic manipulations or pharmaceutical treatments. Several setups for measurement of the zebrafish OKR have been described. All of them are based on the presentation of moving gratings to the larva or to the adult fish. However, they differ in the way of presenting gratings and in the method of analysis. Here, we describe a detailed protocol for our newest software that enables computer-generation of the moving stripes and automatic tracking of eye movement. This protocol makes it possible to quantitatively measure OKR in both larvae and adult fishes in a fast and reliable way.

Key words: Zebrafish larvae, Adult zebrafish, Optokinetic response, Eye movements, Vision, Visual behavior testing, Oculomotor

1. Introduction

Eye movements occur in all vertebrates and in some invertebrates and are thought to be required for high-resolution vision. Two main groups of eye movements exist. Gaze shifting eye movements aim at shifting of the eyes toward an object of interest and include saccadic movements, smooth pursuit, and vergence movements. Gaze stabilizing eye movements include the vestibular ocular reflex (VOR) and the optokinetic response (OKR) and aim at stabilization of a relative movement of the image on the retina, the retinal slip. Retinal slip is caused by either self-motion or motion of the surround and results in a blurred image. VOR and OKR are involuntary compensatory eye movements restoring high visual acuity. When the environment is continuously moving in one direction, the OKR produces a nystagmus composed of cycles of a slow eye

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movement in the direction of the stimulus and a fast resetting movement, called saccade, in the opposite direction. The OKR is triggered by a velocity and direction input coming from the retina and encoded by a neural circuit involving pretectal nuclei (1, 2).

In a laboratory setting, an OKR can be easily elicited by a striped drum rotating around the subject. The OKR has been measured in a number of model organisms, incl. monkey, rabbit, mouse, and fish (e.g., goldfish, medaka, and zebrafish) (3–8). The combination of high-fecundity, extracorporally developing embryos and rapid development of most functions, incl. the visual system, has made zebrafish a model organism of increasing importance for studying visual function. Zebrafish are afoveate animals and therefore, in contrast to humans, do not display gaze shifting eye movements. Another difference between the human and fish visual system is the position of the eyes and the anatomy of the optic nerve. Zebrafish are lateral eyed, and binocular overlap is minimal since all axons from the optic nerve cross at the optic chiasm and project to the contralateral brain side. Humans have frontally positioned eyes and binocular vision, since around half of the axons project to the ipsilateral brain side (2). These differences allow us to study the OKR in zebrafish without the complications of smooth pursuit and binocular vision.

Several setups for measurement of the zebrafish OKR have been described. All of them are based on the presentation of moving gratings to the larva or, more recently, to the adult fish. However, different approaches exist for presenting the gratings and analyzing data. In initial experiments, the larva was placed inside a rotating drum equipped with vertical black-and-white stripes. The rotational speed of the drum was changed mechanically (9). In order to change the properties of the visual stimulus, different drums with stripes of different contrast or width can be used. Although this method is still widely used (10), computer-generated moving gratings are to our mind more convenient, since they allow to continuously change different parameters, such as contrast, spatial frequency and/or angular velocity, direction of rotation, and any other stimulus parameter of choice. In order to project the gratings onto the drum, a digital light projector is placed either on the plane of the subject (linear projection) or below the subject. Using linear projection, only monocular stimulation is possible (11). When the projector is placed below the subject, the gratings are projected via a mirror to the whole drum enabling binocular stimulation (12, 13). In order to avoid visible light from the projector influencing the recording, the animal is illuminated from below with infrared-emitting diodes. An infrared-pass filter in front of the camera ensures selective transmission of the infrared light to the camera.

In initial experiments, analysis of eye movement was performed by visual inspection and by counting the number of saccades occurring. Although this qualitative method—first described for zebrafish by

Clark (14)—has been very convenient for a rapid screening of vision mutants, a quantitative approach is needed for uncovering more subtle oculomotor defects. This has been achieved by computer-based tracking of eye position and subsequent quantitative analysis of changes in eye position over time. For this method—first described by Roeser and Baier (12)—image series are acquired by an infrared-sensitive CCD camera mounted onto a dissecting microscope. Custom-made tracking software extracts information about eye position from the acquired images.

Most OKR setups described in the literature are built for measurement of eye movements in larvae. OKR testing in adult fishes is more challenging, mainly because of the difficulty of restraining body movements of the fish. We were able to solve this problem and published a working method for OKR measurement in the adult (13). In the same year, an alternative setup has been described by Zou et al. (15). In this paper, however, eye movements are only qualitatively analyzed through visual inspection instead of software-based tracking of eye position.

Here, we describe a detailed protocol for the custom-made setup currently used in our laboratory. The animal is stimulated binocularly by computer-generated gratings and the eye position over time is automatically tracked. The resulting eye velocity is calculated in real time. We describe the detailed procedure for recording OKR in larvae as well as in adult fishes. We then describe our standard eye movement quantification approach which allows for detection of subjects with vision defects as well as for investigation of the OKR behavior itself. Since our system is under continuous development (1, 2, 11, 13, 16, 17), some of the details described in the protocol may change over time. However, our detailed protocol should enable the reader to apply the methodology of quantitative OKR measurements. Recently a commercial instrument based on the described setup has become available (VisioTracker by TSE-Systems).

Additionally, we present here a simple assay that enables a nonautomated qualitative analysis of OKR performance in larvae without the need of a computer-based setup. This methodology is suited for those researchers that do not have access to a computer-based setup and are interested in a rapid qualitative screening of vision mutants.

2. Materials

2.1. Reagents

1. 3% methylcellulose in water: Boil 100 ml ddH_2O in a beaker, then start stirring. Add 3 g methylcellulose (while the hot water is stirring vigorously). Continue to stir till the methylcellulose is dispersed into the liquid. Pour the dispersion quickly into two 50 ml Falcon Tubes and rotate (360°) at 4°C overnight. The day after, spin the clear viscous solution at 4°C, 179×*g* for ca.

10 min, in order to remove air bubbles. Store at 4°C for long-term use. Incubate the solution at 28°C for about a day before use (see Note 1).

2. Tricaine methanesulfonate solution (MS-222; Sigma E10521): Dissolve 300 mg Tricaine methanesulfonate in 1 L fish system water.

2.2. Equipment for OKR Recording

1. Serum pipette.
2. Dissecting needle.
3. Forceps.
4. Thin wooden stick.
5. OKR setup for larvae comprising (Fig. 1):
   (a) A dissecting microscope (e.g., SZH-10, *Olympus Corporation*, Japan).
   (b) An infrared-sensitive CCD-camera (e.g., Guppy F-038B NIR, *Allied Vision Technologies*, Germany) equipped

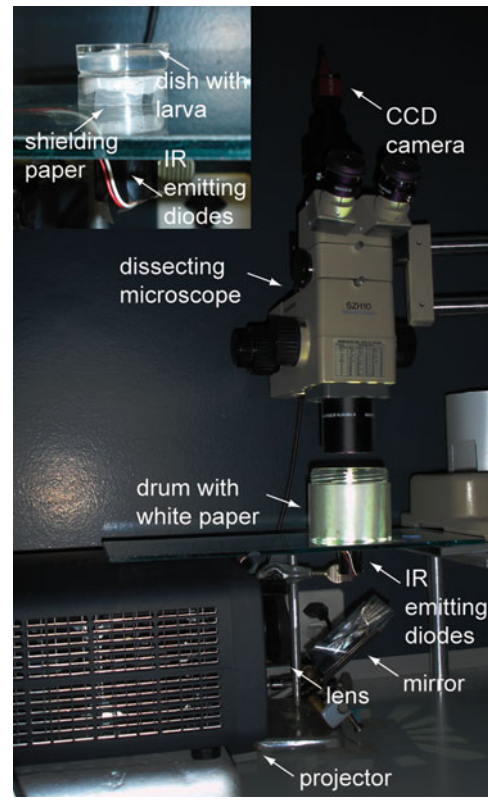

Fig. 1. Setup for the measurement of the OKR in larvae. The computer-generated stimulus pattern is projected via a wide-angle conversion lens to a mirror placed below the larva. The stimulus is reflected in the mirror and directed onto a drum surrounding the larva. A cluster of 15 infrared-emitting diodes illuminates the larva from below and is shielded by a piece of wax paper in a 35 mm Petri dish (see inset on the *left*). An infrared-sensitive CCD camera on the top of a dissecting microscope records the movement of the eyes.

with an infrared-pass filter (e.g., RG715, *Olympus Corporation*, Japan).

(c) A glass plate as a stand for the animal and the drum.

(d) A stimulus computer running the open source Python library Vision Egg (18).

(e) An LCD projector (e.g., PLV-Z3000, *Sanyo*, Japan) (see Note 2).

(f) A wide-angle conversion lens (e.g., HD-4500PRO, *Raynox*, Japan).

(g) A mirror.

(h) A control computer running custom-made software based on NI LabView 2009 and NI-Vision Development Module 2009 (*National Instruments*, USA).

(i) A cluster of infrared-emitting diodes ($\lambda_{peak} = 940$ nm) (e.g., BL0106-15-28, *Kingbright*, Taiwan) shielded by a piece of wax paper in a 35 mm Petri dish.

(j) 35 mm Petri dish containing the larva embedded in 3% methylcellulose and aligned to lay dorsal side up.

(k) A transparent plastic drum containing a white blotting paper on its internal wall.

6. OKR setup for adult fishes: (a) to (i) are identical to the setup for larvae. Additionally, the setup for adult fishes comprised the following:

(j) A custom-made glass chamber ($W \times H \times L = 12$ mm $\times 12$ mm $\times 65$ mm) (Fig. 2) containing the fish restrained by two pieces of sponge and two plastic half pipes. Two inlets attached to both sides of the chamber allow for fish water inflow. A third tube attached at the end of the chamber allows for water outflow back to the supply tank.

(k) A support stand.

(l) A peristaltic pump (e.g., SR25, 65 rpm, 24 VDC, novoprene tube N 4.8 mm $\times$ 1.6 mm, *Gardner Denver Thomas*, USA).

(m) A 24 V power supply for the pump (e.g., FSP 2405, *Voltcraft*, Germany).

(n) A USB-Relais to switch ON/OFF the pump (e.g., USBREL8, *Quancom Informationssysteme GmbH*, Germany).

(o) A water bath equipped with an aquarium heater (e.g., 50 W, *Jäger*, Germany).

(p) An air pump (e.g., R301, *Rena*, USA).

(q) A white plastic drum ($d = 12.5$ cm; e.g., cut from a chemical drum) with three small openings at the bottom edge for the tubes of the flow-through chamber.

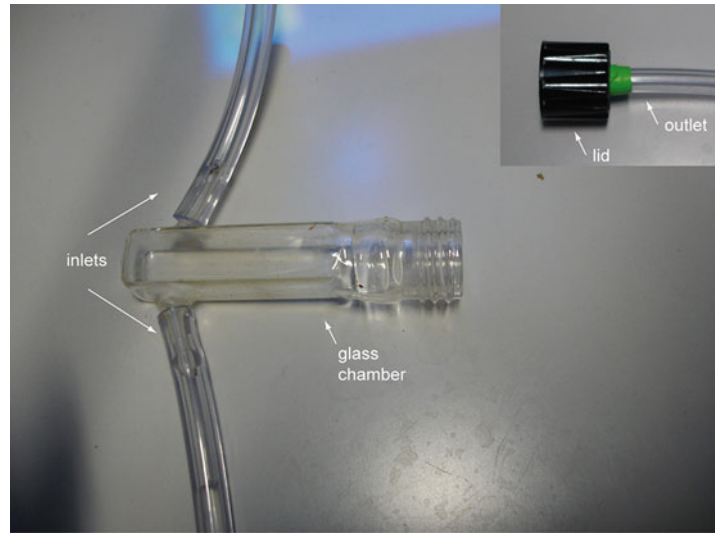

Fig. 2. Custom-made flow-through chamber to restrain the fish. The fish is restrained as described in methods. Fish water—maintained at 28°C in a water bath and oxygenated by an air pump (not shown)—flows at max 40 ml/min on the gills through two inlets attached to both sides of the chamber. The flow rate is generated by a peristaltic pump (not shown). The water effuses back to the supply tank via a third tube attached on the lid of the chamber.

2.3. Equipment for Manual OKR Measurements (Fig. 3)

1. Dissecting microscope (e.g., SV8, *Zeiss*, Germany).
2. Light source with light guides (e.g., KL 750, *Leica*, Germany).
3. 35 mm Petri dish containing the larva embedded in 3% methylcellulose and aligned to lay dorsal side up.
4. Turntable (turning can be done manually or by a motorized drive).
5. Paper with stripes of the desired color and width. The paper has to fit in the turntable.
6. Serum pipette.
7. Dissecting needle.

3. Methods

3.1. Recording of the OKR in Larvae

Protocols for fish breeding can be found online in the Zebrafish book (http://zfin.org/zf_info/zfbook/zfbk.html) or in *Zebrafish: A practical approach* (19).

3.1.1. Embedding the Larva (See Note 3)

1. Pour pre-warmed (28°C) 3% methylcellulose solution in a 35-mm Petri dish. Be careful not to produce air bubbles (see Note 4).
2. Suck a larva with a serum pipette and put it on the methylcellulose together with as little E3 medium as possible. To achieve

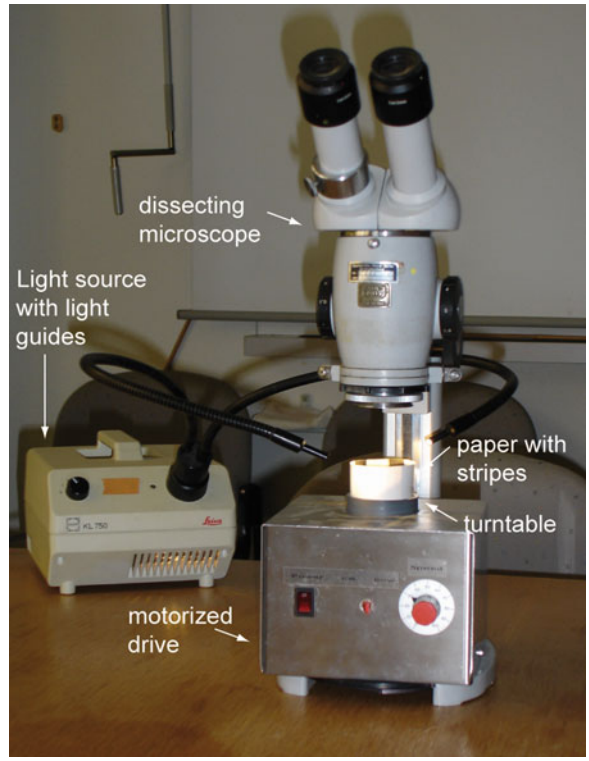

Fig. 3. Setup for manual measurement of the OKR in larvae. The larva is placed on a turntable inside of a paper with a striped pattern. Rotation of the drum is driven by a motorized drive. The larva is illuminated from above by a light source with light guides. The eye movement is observed through the dissecting microscope.

this, we tap the side of the pipette so that the larva swims to the bottom. Suck off any remaining E3 medium around the larva in order to avoid dilution of the methylcellulose solution.

3. Embed the larva dorsal up in the center of the dish. To orient the larva use a dissecting needle (see Note 4).
4. Allow the larva to get used to the methylcellulose for about 10 min before starting recording.

3.1.2. Starting the Setup

1. Start up the whole setup:
   (a) Switch on both the stimulus and the control computer.
   (b) Plug in the infrared LED-cluster
   (c) Switch on the projector.
2. Write the Configuration File containing the stimulus parameters (see Notes 5–7).
3. Stimulus computer: Start the stimulus program and wait for a message-box. Press "Bind port and listen for connections."
4. Control Computer: Start the OKR program. Press "New set up larvae." The OKR user interface will appear on the screen (Fig. 4).

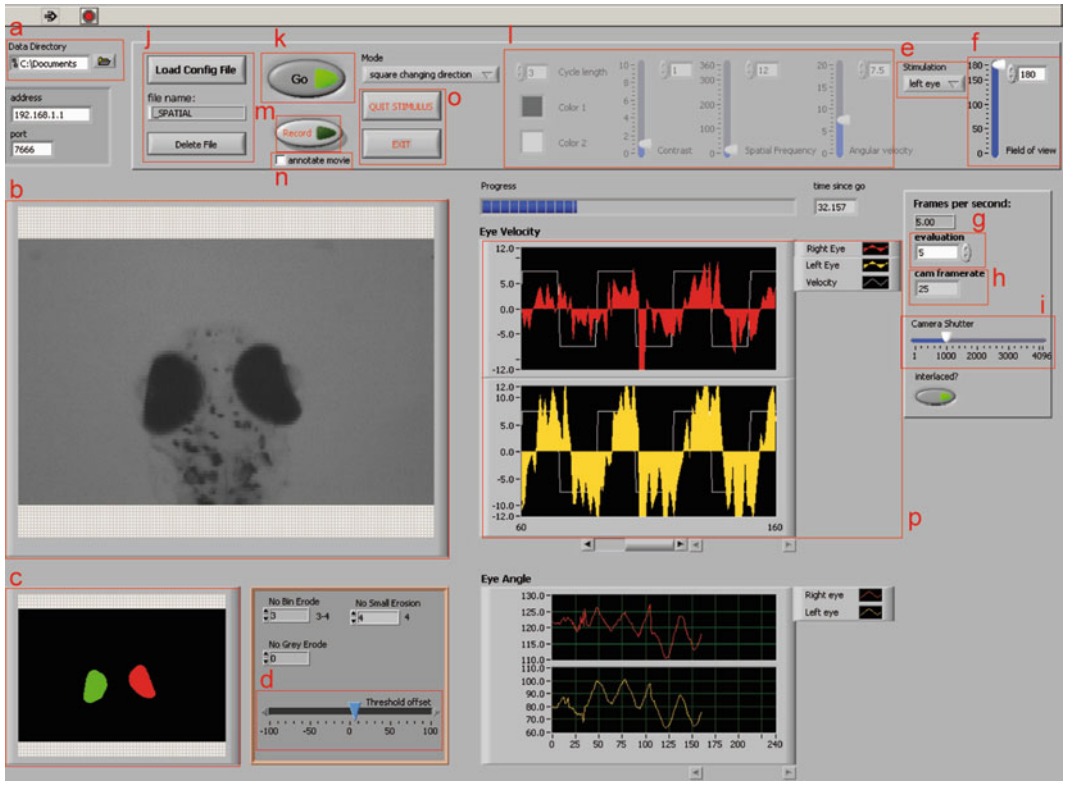

Fig. 4. OKR user interface for eye movement measurement in larvae. Recording controls are on the *top*. A real-time image of the larva is displayed on the *left*. Tracking and eye velocity data are shown in the *center*. The angle and the velocity of the right and left eye are displayed, the velocity of the rotating pattern is shown with a *white line*. On the *bottom left* are the particle detection parameters. On the *right* is the control of frame-rate. Letters (a) to (p) refer to the steps described in the main text.

3.1.3. Recording Eye Movement

1. Choose the data folder where you want to save your data (a). Then press "Current folder."
2. Place the larva under the dissecting microscope and center it in the visual field of the camera (b). The larva should be oriented in the same direction as the light beam. On the screen the larva is seen as in Fig. 4 (see Note 8). Choose the highest possible magnification. Pay attention that the eyes are visible on the screen. When the larva is in focus, place the plastic drum around the animal.
3. The software recognizes the dark pigmented eyes based on the pixel intensity. Check if the eyes are recognized well (c), and adjust the "threshold offset" (d) if necessary (e.g., if body pigmentation spots are close to the eye).
4. Choose between a binocular stimulation (field of view = 360°), a monocular stimulation of the right eye, and a monocular stimulation of the left eye (e). In the case of a monocular stimulation, the field of view can be regulated (between 0° and 180°) (f).

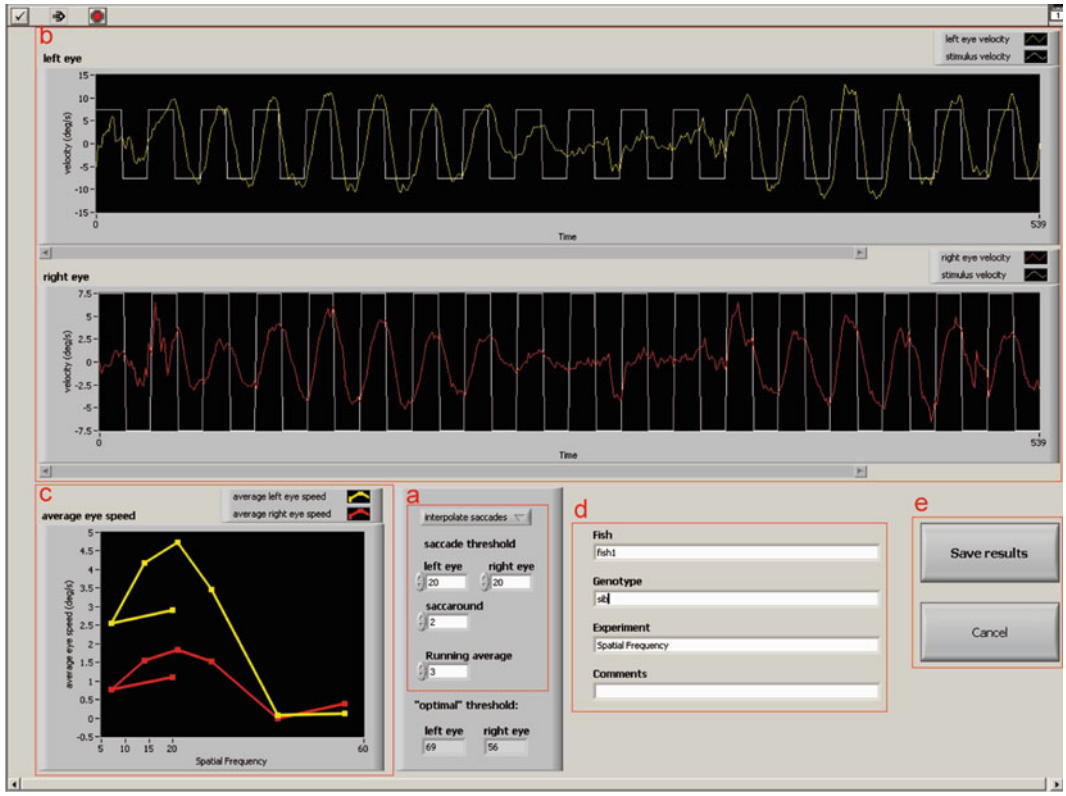

Fig. 5. User interface for smoothing and saving the data. On the *bottom right* the parameters to filter saccades and smoothen the velocity curves can be set. The effect of these changes is seen on the velocity curves on the *top* and on the velocity averaged over the same stimulus conditions (*bottom left*). Letters (a) to (e) refer to the steps described in the main text.

5. Choose the frame rate at which the images from the camera are processed by the software (g). We use 5 frames/s for screening of mutants and 25 frames/s for quantitative analysis of the OKR behavior itself. This frame rate has to be lower than the frame rate of the camera (h). Change the opening time of the camera shutter if necessary (i). Lowering the opening time reduces image brightness but increases the frame rate (h).
6. Load the Configuration File (j).
7. Press the "Go"-button (k) to start the experiment (see Notes 9 and 10). The experiment can be aborted by pressing "Go" again. If "Go" is pressed without having loaded a Configuration File, the stimulus will run with the parameters shown in (l). These parameters (colors, contrast, spatial frequency, and angular velocity) can be changed here. However, without a Configuration File the eye position over time will not be recorded.
8. When the end of the Configuration File is reached, a window appears (Fig. 5). Here, the parameters to filter saccades and smooth the velocity curves—saccade threshold, saccaround,

and running average—can be set (a) (see Note 11). The velocity curve of each eye after smoothing is shown in (b) and the velocity averaged over the same stimulus conditions is indicated in (c). Enter subject information (fish number, genotype, experiment, and, optionally, any comments) (d). Save the results (e).

9. After the first run as well as after having changed the Configuration File, a window pops up with the request to enter the name for a results-file or to choose an already existing one. Enter a name or choose an existing file. As long as the Configuration File is not changed, the following recordings will be saved in the same results-file. The results-file contains values for the average slow-phase velocity for each fish and for each measured conditions. For each fish recorded, an additional tab-file containing the raw data is automatically saved. Each line represents a frame. Columns A and B contain values for the angular eye position of the right and the left eye, respectively. Columns C and D contain values for the eye velocity in degree per second of the right and the left eye, respectively. The further columns contain information about the stimulus parameters.
10. Continue with step 4 to measure the same larva with a new paradigm. Go back to step 2 to measure a different larva.

3.1.4. Recording a Movie

All the frames imaged by the camera during stimulus presentation can be recorded and visualized later on.

1. Before starting the stimulus, press the button "Record" (see Fig. 4, (m)).
2. Enter the name under which the movie has to be saved. Movies are automatically saved in AVI-format.
3. Activate "annotate movie" (n) if it is wished that the current stimulus properties are written in the lower right corner of each frame.
4. Start the stimulus as described above.
5. Press "Record" (m) again to stop recording of the movie.

3.1.5. Shutting Down the Setup

1. Press "Quit Stimulus" and "Exit" (see Fig. 4, (o)).
2. Shut down both computers.
3. Unplug the IR LED-Cluster.
4. Switch off the projector.

3.2. Recording of the OKR in Adult Fishes

3.2.1. Starting the Setup

1. Step 1 is identical as for the setup for larvae (see Subheading 3.1.2).
2. Write the Configuration File containing the stimulus parameters (see Note 12).

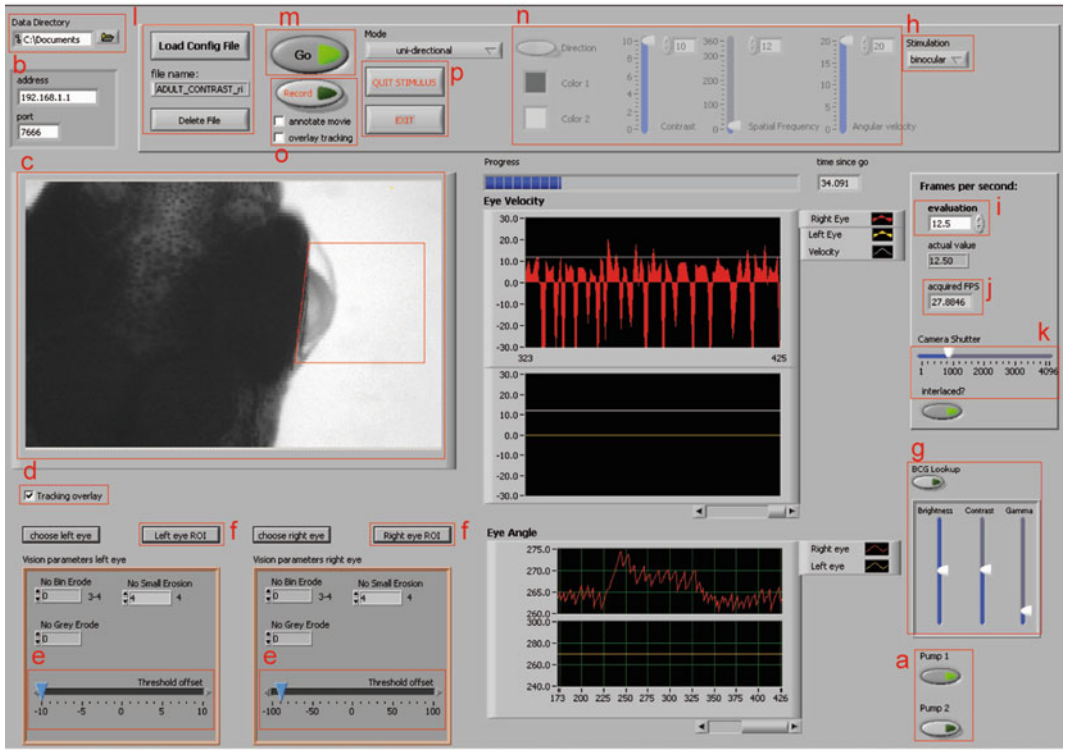

Fig. 6. OKR user interface for eye movement measurement in adult. Recording controls are on the *top*. A real-time image of the fish including particle detection is displayed on the *left*. On the *bottom left* are the particle detection parameters. Tracking and eye velocity data are shown in the *center*. On the *right* is the control of frame rate. Letters (a) to (p) refer to the steps described in the main text.

3. Stimulus computer: Start the stimulus program and wait for a message-box. Press "Bind port and listen for connections."
4. Control Computer: Start the OKR program. Press "New set up adults." The OKR user interface will appear on the screen (Fig. 6).

3.2.2. Restraining the Fish

1. Warm up fish water in the supply tank using a water bath set at 28°C. Oxygenate the fish water with an air pump.
2. Turn the flow-through chamber to a vertical position (front end down) and fill it with fish water by switching the pump on the user interface (see Fig. 6 (a)) until the water level reaches the upper rim.
3. Briefly anesthetize the fish in 300 mg/l MS-222 (see Note 13).
4. Prepare a half plastic pipe and insert a humid piece of sponge.
5. As soon as the fish stops swimming, gently lay the body on the piece of sponge, leaving the head incl. the gills free (Fig. 7a).
6. Cover with a second humid piece of sponge (Fig. 7b) and stabilize the sponges with a second half plastic pipe (Fig. 7c).

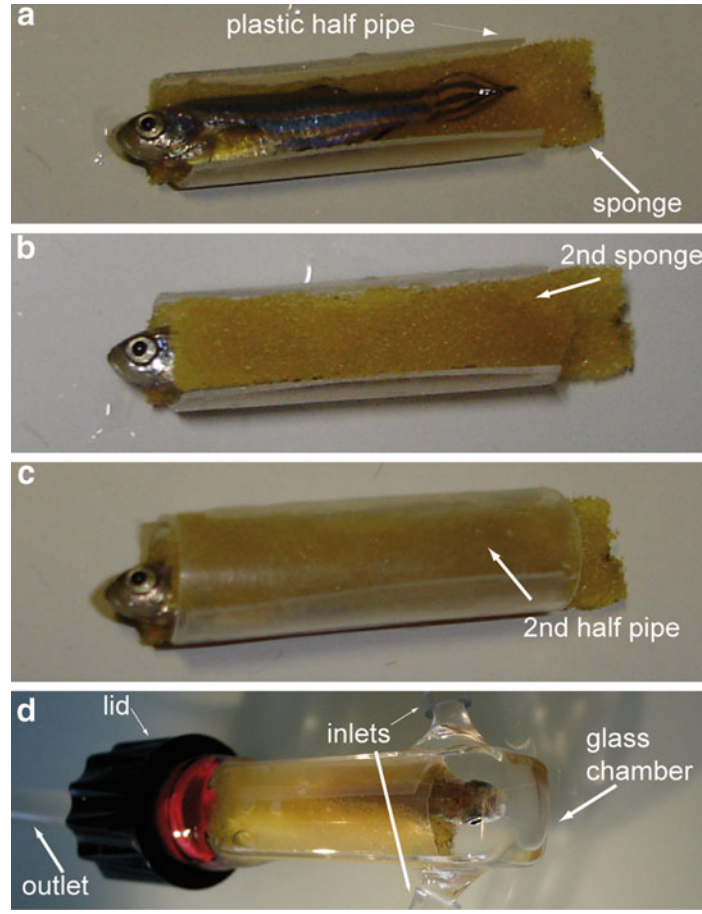

Fig. 7. Steps for restraining an adult fish. (**a**) The anesthetized fish is laid on a humid piece of sponge, which had been inserted into a plastic half pipe. (**b**) The fish is covered with a second humid piece of sponge. (**c**) Everything is covered with the second half of the plastic pipe. (**d**) The restrained fish is inserted into the glass chamber that had been connected to the two inlets and filled with fish water. The head of the fish looks to the bottom of the chamber. The chamber is then closed with the lid, which is attached to the outlet.

Again, pay attention to leave the head and the gills free (see Note 14).

7. Fit everything into the flow-through chamber which is fixed on a support stand. The fish has to look toward the bottom of the chamber (Fig. 7d). Use a thin wooden stick to push the fish together with the pieces of sponge and plastic half pipes down until the gills are on the height of the water inlets. Take care that no air bubbles are present in the front end of the chamber, i.e., around the head of the fish.
8. Close the lid of the flow-through chamber with the water outlet attached.
9. Switch on the peristaltic pump.

3.2.3. Recording Eye Movements

The setup for the adult is similar to the larval one.

1. Choose the data folder where you want to save your data (Fig. 6, (b)). Then press "Current folder."
2. Turn the flow-through chamber containing the fish into horizontal position, place it under the dissecting microscope, and center it in the visual field of the camera (c) (see Note 15). The fish should be oriented in the same direction as the light beam. Choose an appropriate magnification (the eye to be recorded from should be as large as possible to still fit into the image) (see Note 16). Place the plastic drum around the fish such that the three tubes of the chamber can exit the drum through its openings.
3. In the setup for adults the particle detection is directly overlaid on the live image (c) if "Tracking overlay" is activated (d). Select a ROI around the lens of the eye to be recorded from by pressing "Right eye ROI" or "Left eye ROI" (f). Check if the rim of the eye is recognized well (c) and adjust the "threshold offset" for the eye to be recorded (e).
4. Contrast, brightness, and gamma of the image can be adjusted after having activated the button "BCG Lookup" (g).
5. We usually stimulate adult fishes binocularly (field of view = 360°). However, it is possible to choose a monocular stimulation of the right eye and a monocular stimulation of the left eye (h). In the case of a monocular stimulation, the field of view can be regulated as in larval experiments.
6. Choose the frame rate at which the images from the camera are processed by the software (i). We typically use 12.5 frames/s. This frame rate has to be lower than the frame rate of the camera (j). Change the opening time of the camera shutter if necessary (k).
7. Load the desired Configuration File (l).
8. Press the "Go"-button (m) to start the experiment. The experiment can be aborted by pressing "Go" again (see Notes 17–19). If "Go" is pressed without a Configuration File loaded, the stimulus will run with the parameters shown in (n) as in the setup for larvae.
9. When the end of the Configuration File is reached, the data can be filtered and saved as in the setup for larvae (Fig. 8 and Note 20).
10. The same fish can be measured again with a new paradigm. Fishes easily survive for 30 min in the chamber without consequences on their health.
11. After successful measurement, turn the chamber back to a vertical position, switch off the pump, and remove the fish together with the sponge and plastic half pipes using forceps. Release

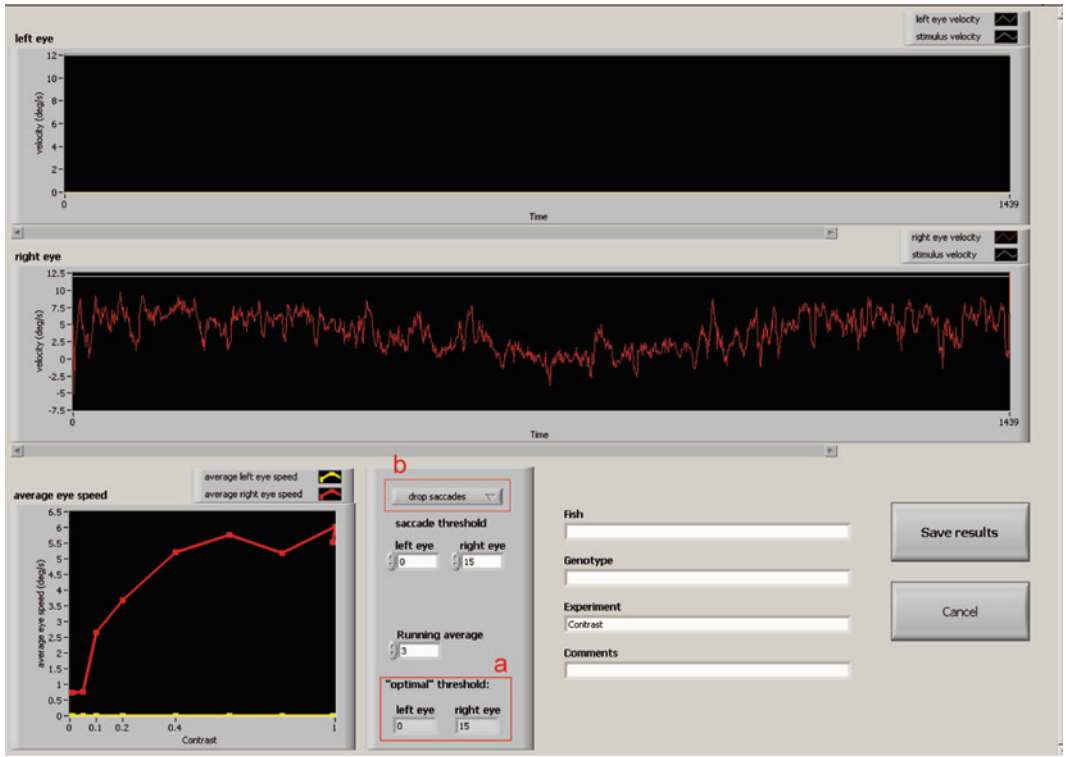

Fig. 8. User interface for smoothing and saving the data in the adult fish. On the *bottom right* the parameters to filter saccades and smoothen the velocity curves can be set. The effect of these changes is seen on the velocity curves on the *top* and on the velocity averaged over the same stimulus conditions (*bottom left*). Letters (a) and (b) refer to the steps described in the main text.

the fish into a tank with fish water. Fill the chamber with fish water again by switching the pump on until the water level reaches the upper rim. Continue with Subheading 3.2.2, step 3, to measure the next fish.

3.2.4. Recording a Movie

A movie of the live image can be recorded as with larvae (see Fig. 6, (o)). Activate "overlay tracking" to overlay the eye tracking on each frame.

3.2.5. Shutting Down the Setup

1. Switch off the peristaltic pump.
2. Remove the fish from the chamber and put it back to its tank.
3. Shut down the aquarium heater, the power supply of the pumps, and the air pump.
4. Press "Quit Stimulus" and "Exit" (see Fig. 6, (p)).
5. Shut down both computers.
6. Unplug the IR LED-Cluster.
7. Switch off the projector.

3.3. Quantification of Eye Movement

Here, we describe the quantification method currently most used in our laboratory. This method is well suited for screening of vision defects. A more precise method for investigation of the OKR itself has been recently developed in our laboratory (16) (see Note 21). However, since investigation of the OKR itself is probably not the aim of most readers, we do not go through the details at this place.

3.3.1. Plotting the Average Eye Velocity over an Experimental Condition

We usually plot the average slow-phase eye velocity over an experimental condition, e.g., varying contrast. For this, we use the automatically generated results-file which contains the average slow-phase velocity for each condition and for each subject. This value has been calculated after filtering and smoothing of the raw data. Data analysis can be performed with any statistics software.

1. Open the results-file with the statistical program of preference.
2. Plot a line graph with the varying condition on the *x*-axis and the average eye velocity on the *y*-axis. If different groups need to be compared (e.g., different genotypes or different treatments), plot them as different series in one graph.

3.4. Manual OKR Measurements

Larvae are embedded in 3% methylcellulose as described for OKR recording (see Subheading 3.1.1).

3.4.1. Embedding the Larva

3.4.2. Measuring of the OKR

1. Insert the striped pattern of choice in the turntable to form a drum.
2. Place the larva inside the drum.
3. Switch on the light source.
4. Rotate the drum and watch eye movements through the microscope.
5. Score the direction of slow-phase movements (with the drum or reverse to the drum movement). Count the number of saccades per given time interval as a readout of performance (see Note 22).

4. Notes

1. Methylcellulose is difficult to solubilize. When methylcellulose is added to hot stirring water, a cloudy dispersion is formed. This takes 1–2 min. Afterwards, the dispersion has to be poured very quickly into Falcon tubes to avoid sedimentation of the methylcellulose on the bottom of the beaker. This would give rise to aliquots of different concentrations. The Falcon

tubes need to rotate as soon as they are at 4°C because the methylcellulose starts to solubilize quickly at this temperature. The day after, the solution has to be centrifugated till all air bubbles disappear. We recommend to keep the solution at 4°C for long-term storage. However, the methylcellulose has to be warmed up to 28°C before use. This is the protocol used currently in our laboratory and is based on Brockerhoff et al. (10). However, other protocols exist and may work as well (19).

2. Resolution of the projector should be as high as possible (preferably use an HD-projector) to enable presentation of narrow stripes necessary to determine spatial resolution. In addition, the projector should have a high contrast ratio and a deep black level.
3. We embed our larvae in a 3% methylcellulose solution in order to restrain body movement with only minimal effect on eye movements. Zebrafish larvae survive in methylcellulose since this is a nontoxic viscous medium that allows oxygenation through the skin. Alternatively, the body of the larva can be embedded in low-melting agarose with the head and gills exposed to water as described by Beck et al. (20). However, this method is more time consuming.

   Dishes containing methylcellulose can be reused several times, as long as the quality is intact (air bubbles should not be present; the solution should not be diluted or too sticky). In order to recycle them, dishes can be stored at 28°C in a humidified chamber for later use.
4. Avoid production of air bubbles at any time point by gently pouring the methylcellulose solution into the dish and by gently positioning the larva inside the solution.
5. To write a Configuration File, open an empty excel datasheet. Each column represents one parameter, and each line one sequence. A new sequence needs to be started as soon as one parameter changes. Enter the parameters as described below and save the file as a tab-file (see Fig. 9 for an example):
   (a) Column A: Write “Contrast” in the first line. For sine-wave grating choose values between 0 and 1. If you want sharp stripes with sharp borders, choose 10. Write a new line for each new sequence.
   (b) Column B: Write “Spatial Frequency” in the first line. The Spatial Frequency (SF) is given in cycles/360° and determines how many patterns of two different stripes are displayed in 360° (e.g., a value of 1 means that 2 stripes with two different colors are shown). Choose the desired value for each sequence. To determine the visual acuity of a larva, we normally run sequences with values between 7 and 56.

	A	B	C	D	E	F	G	H	I	J	K
1	Contrast	Spatial Frequency	angular velocity	Color1 red	Color1 green	Color1 blue	Color2 red	Color2 green	Color2 blue	No. Cycles	Cycle duration
2	0.7	20	7.5	0.6	0.6	0.6	0	0	0	3	3
3	0.7	7	7.5	0.6	0.6	0.6	0	0	0	3	3
4	0.7	14	7.5	0.6	0.6	0.6	0	0	0	3	3
5	0.7	21	7.5	0.6	0.6	0.6	0	0	0	3	3
6	0.7	28	7.5	0.6	0.6	0.6	0	0	0	3	3
7	0.7	42	7.5	0.6	0.6	0.6	0	0	0	3	3
8	0.7	56	7.5	0.6	0.6	0.6	0	0	0	3	3
9	0.7	42	7.5	0.6	0.6	0.6	0	0	0	3	3
10	0.7	28	7.5	0.6	0.6	0.6	0	0	0	3	3
11	0.7	21	7.5	0.6	0.6	0.6	0	0	0	3	3
12	0.7	14	7.5	0.6	0.6	0.6	0	0	0	3	3
13	0.7	7	7.5	0.6	0.6	0.6	0	0	0	3	3

Fig. 9. Example of Configuration File. In this example SF is changed in each sequence, contrast and angular velocity are constant. The first line represents the calibration sequence (see Note 7).

(c) Column C: Write "angular velocity" in the first line. This parameter determines the angular velocity of the stimulus and is given in degree per second. Choose the desired value for each sequence. To determine temporal resolution, we typically run sequences with values between 5 and 30.

(d) Column D–F: Always in the first line, write "color1 red" in column D, "color1 green" in column E, and "color1 blue" in column F. Choose values between 0 and 1. Each value specifies the intensity of the respective color-channel. For grey stripes choose the same value for all channels, whereby the value has to be higher than 0 and smaller than 1. We routinely use a value of 0.6. For completely white stripes use a value of 1 for all channels.

(e) Column G–I: Analog to column D–F but for color 2. For black stripes choose the value 0 for all channels. For pure red set green and blue to 0 and red to 1.

(f) Column J: Write "nr Cycles" in the first line. Choose the number of cycles needed for each sequence. A value of 2 means that the stimulus will change the direction of rotation once during the specific sequence.

(g) Column K: Write "Cycle duration" in the first line. It defines the duration of each cycle in seconds. Choose the value wanted for each sequence.

6. We normally change only one parameter in each Configuration File, e.g., we measure the contrast sensitivity and therefore vary the contrast value but leave all other parameters constant.

In the case of contrast, we start with the highest contrast, reduce it stepwise, and increase it again. Note that the contrast values from 0 to 1 are relative with 1 being the maximal contrast chosen. The real contrast has to be determined by measuring the luminance from the drum with a photometer.

In the case of SF and angular velocity, we start with the lowest value, enhance it stepwise, and reduce it again.

7. At the beginning of recording, the eyes are pre-stimulated with a standard stimulus (typically contrast = 0.99, SF = 20 cycles/360°, and angular velocity = 7.5°/s for larvae). This avoids artifacts from starting the experiment. This pre-stimulation is written as the first sequence in the Configuration File and should last typically for 9 s. Data from this sequence will be deleted before analysis.
8. Sometimes the larva is not immobilized properly. In this case it may help to wait longer till the start of the recordings. The larva will eventually calm. A drift of the larval position over time could be due to movement of the viscous solution because of handling. Also in this case the drift should reduce over time. It also helps to use light-adapted larvae if this is compatible with the experiment as light-adapted larvae tend to be calmer. If the larva is still moving, please check the following:
   (a) Make sure that the larva is embedded dorsal side up.
   (b) Check the texture of the methylcellulose solution. If it is too diluted, try with a new solution.
   (c) If the mutation/treatment analyzed causes a higher motor activity, it may be necessary to increase the methylcellulose concentration.
9. If the eye movement is low or absent check the following:
   (a) Make sure that the larva is still alive by checking its blood flow.
   (b) Make sure that the stimulus is running properly. If the stimulus shuts down unexpectedly, close the software and the python program and restart both (first the python program and then the OKR software).
   (c) Make sure that the projector lamp is working properly and not getting weaker. Measure the luminance from the drum during stimulus presentation using a photometer. We recommend to do this on a regular basis, at least every 6 months, in order to assure that contrast and brightness stay constant over time.
   (d) Look for light sources in the room that could interfere. Maintain the room as dark as possible.
   (e) Check the quality of the methylcellulose solution.
   (f) Make sure that the larva is embedded dorsal side up.
   (g) Measure a healthy and untreated wild-type larva as a control. If this larva shows a normal OKR and you have checked all points from (a) till (f), you may have found a larva with impaired OKR. Congratulation!
10. Sometimes eye movement does not seem to be matched to movement of the stimulus (see Fig. 4, (p)). If this happens, make sure that the stimulus runs stably. Check for irregularities

in stimulus pattern velocity and check for any deviance from the parameters determined in the Configuration File. If deviances are present, restart the python file and then the OKR software.

11. The eye velocity is determined from the eye position over time. We usually consider the eye velocity during the slow phase of the OKR (SPV) as a readout for OKR performance. In order to calculate the SPV, we need to filter out the saccades (fast resetting movements in the opposite direction than the stimulus) and to smooth the curve. We usually do this with the help of an empirically tested formula (17): If eye velocity (v) in a certain frame (f) exceeds a determined saccade threshold (default: 20°/s), eye velocity of this frame as well as of a defined amount of preceding frames (saccaround) is replaced with the eye velocity of the frame preceding the saccaround. Analogously, the eye velocity of the defined amount of following frames is replaced by the value 1 frame after the saccaround. By a frame rate of 5 frames/s, we usually set the saccaround to 2 (($vf…f-2$) is set to $v(f-3)$ and ($vf+1…f+2$) is set to $v(f+3)$). The velocity curve is further smoothened by a running average. At a frame rate of 5 frames/s, we usually set a running average of 7 frames ($v(f)=(\Sigma v(f-3…f+3))/7$). It is also possible to drop the saccades without saccaround. This can be defined in (a) on the top (see Fig. 5). See Subheading 3.3 and Note 21 for more details on data analysis.
12. Write the Configuration File following the guidelines for experiments with larvae (see Notes 5–7). For recordings in adult fish, we typically stimulate binocularly and in one direction only. Therefore, each sequence consists of only one cycle. The length of the sequences can be set as preferred. We usually record with sequences lasting for 9 s. As for recordings in larvae, eyes are pre-stimulated with a standard stimulus typically lasting 9 s with contrast = 0.99, SF = 36 cycles/360°, and angular velocity = 12°/s. This pre-stimulation is not considered in data analysis.

    To determine the visual acuity of an adult fish, we usually run sequences with SF values between 18 and 180 cycles/360°. To determine the temporal resolution, we usually run sequences with angular velocity values between 5 and 55°/s.
13. Always use a freshly prepared solution of MS-222, since tricaine is light sensitive and quickly loses its activity, and toxic by-products may be formed.
14. In case fishes strongly vary in size, use different pieces of sponge with different sizes, or add additional small pieces for smaller fish.
15. Before initiating an experiment, leave the fish in the flow-through chamber for 1–2 min with running water supply in order to let it recover from anesthesia and calm down.

16. Since temporal-to-nasal eye velocity has been shown to be much higher and more stable (13), we usually evaluate only the eye stimulated in temporal-to-nasal direction. This way we can also control the position of that eye more precisely.
17. If eye movements are jerky and not correlated to visual stimulation, stop presentation of gratings and wait for 30 s. Restart the stimulation with optimal parameters, i.e., high contrast (1 or higher), medium spatial frequency (ca. 36 cycles/360°), and high angular velocity (ca. 20°/s). Repeat this until eye movements are stable and well correlated to visual stimulation.
18. If the fish does not show any eye movements at all, make sure that the pump is running. Oxygenation may be insufficient if the gills are covered by the sponge. In this case, immediately release the fish and let it recover in a tank with fresh fish water. Turn the chamber back to a vertical position, switch off the pump, and remove the fish together with the sponge and plastic half pipes using forceps.
19. If the fish manages to disengage itself from the restraining system, shut down the pump, turn the chamber back to a vertical position, open the lid, remove sponge and plastic half pipes using forceps, position a tank with fish water below the chamber, and remove the fish by turning the chamber by 180°.
20. In contrast to the method used for larvae, the threshold for saccade filtering is not fixed but an ideal threshold is searched for each eye in an iterative process. The ideal threshold is the one that results in the highest sum of average eye velocities and it is displayed below the smoothing settings (see Fig. 8, (a)). Moreover, saccades are usually dropped and saccaround is not performed. Nevertheless, it is possible to use the saccaround method. To define the method of choice, press (b). The curve is smoothened by a running average as in recordings of larvae. At a frame rate of 12.5 frames/s, we typically use a running average of 7 (see Note 11 for details about the smoothing algorithm).
21. In our laboratory, different processing methods have been applied in the past depending on the research question (11, 13, 16, 17). Here, we describe in detail the method of choice for a rapid screening of vision defects. However, for a quantitative analysis of the OKR behavior itself—e.g., for analysis of the eye movement waveform—a higher frame rate is needed and the method described here is not precise enough. For this kind of quantitative analysis, we refer to our work on the mutant *belladonna* (16). A fraction of the homozygous *belladonna* larvae displays a reversed OKR and spontaneous eye oscillations in the absence of a moving stimulus. In order to quantitatively analyze those eye movements, a more precise quantification software was developed using the R statistical computing language. Briefly, the eye movement was recorded

with a frame rate of 12.5 frames/s (nowadays we record with 25 frames/s). The eye position trace was smoothened with a Gaussian smoothing kernel. Slow-phase segments were determined by setting acceleration thresholds. The slow-phase velocity was defined by taking the maximum eye velocity across all slow-phase segments within a condition.

22. If the eye movement is low or absent check the following:
    (a) Make sure that the larva is still alive by checking its blood flow.
    (b) Check the light intensity from the light source and try to vary it.
    (c) Look for light sources in the room that could interfere. Maintain the room as dark as possible.
    (d) Check the quality of the methylcellulose solution.
    (e) Make sure that the larva is embedded dorsal side up and calm.
    (f) Make sure that the drum is rotating smoothly.
    (g) Measure a healthy and untreated wild-type larva as a control. If this larva shows a normal OKR and you have checked all points from (a) till (f), you may have found a larva with impaired OKR.

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