Chapter 7

Optogenetic Interpellation of Behavior Employing Unrestrained Zebrafish Larvae

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Abstract

The zebrafish larva, *Danio rerio*, provides superb genetic access for studying how systematic variations in behavioral profiles relate to differences in brain activity. Larvae respond predictably to various sensory inputs and their nervous system is readily accessible. Also, their transparent body allows for noninvasive optogenetics and their small size allows for measuring behavior with full environmental control. In tethered larvae, neural activity has been correlated to eye and tail movements. The challenge now is to tackle the building blocks of behavior: internal states (maturation and learning), motivations (drives), reversible phenotypic adaptations (humoral actions), and decision processes (choice and task selection). These phenomena are best addressed through the analysis of freely behaving subjects. This chapter provides the basics for applying optogenetics to the analysis of behavior in freely swimming larvae. As a study case, we offer information from recent tests showing how optogenetic manipulation of hormone-producing cells can be used to address reversible phenotypic adaptations. Because larvae are highly reactive to optic stimuli, light control is pivotal in employing noninvasive optogenetics. This point is covered in detail, starting from the general rules of light delivery and maintenance prior to the tests.

Key words Optogenetics, Larval zebrafish, Behavior, Photoactivated adenylyl cyclases, Stress

1 Introduction

Caenorhabditis elegans and *Danio rerio* are widely used for studying the neuronal bases of behavior. In recent years, research on both organisms has seen an increasing number of optogenetic tools. *C. elegans*, a transparent nematode, has a nervous system of 302 neurons whose wiring diagram has been established [1]. In combination with easily quantifiable movements serving responses to various sensory inputs and simple forms of learning, *C. elegans* provides a productive ground for the analysis of locomotion patterns [2–4]. It was the first organism in which the microbial rhodopsin, ChR2, was implemented for remote locomotion control [5]; numerous photo-switchable actuators and reporters have

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subsequently been used to link the activity of neural circuits to locomotion schemes [6] (*see* Chap. 6).

Due to their genetic amenability and transparent body, larval zebrafish have also become popular for studying the neuronal bases of behavior. They show a range of locomotion patterns and reactions [7–10], visual reflexes [11, 12], and prey capture movements [13–15]. In tethered larvae, brain regions [16–20] and circuit motives regulating locomotion have been identified via genetically encoded calcium indicators (GECIs) [21–23] (*see* also Chap. 9). Photo-switchable actuators have been used to either increase or decrease neuronal activity [24]. Examples include the use of LiGluR or ChR2 in spinal cord neurons [25, 26], halorhodopsin or ChR2 in hindbrain neurons [27–30], and ChR2 in midbrain (nMLF) [31], pretectal, and tectal neurons [32]. In sum, in combination with high-resolution in vivo imaging and optogenetics, tethered larvae have provided a great deal of help to link brain activity to principles of locomotion control. The analysis of complex behavior, however, cannot be accurately carried out employing restrained subjects. Behavioral complexity rests on the joint work of four regulatory components functionally defined: internal states, motivations, adaptive responses to the environment, and decision processes. To tackle these fundamentals, records of freely behaving subjects will be compulsory.

1. *Internal states* are measured through novelty responses. When released into a novel environment, e.g., animals initially show signs of fear and may remain motionless. This is often followed by exploratory movements. The probability that a given novel situation will elicit exploration rather than quiescence depends on the animal's internal state. Animals that have recently had a stressful experience are more likely to be wary of novel environments.
2. *Motivation* is a reversible aspect of an animal's state that plays a causal role in behavior. Such state is made up of both internal and external factors relevant to incipient activities as well as the animal's current behavior. It follows that motivations can only be studied through the analysis of the relative importance of different activities. To determine motivations, therefore, a subject should be free to respond to local environments with a full repertoire of actions.
3. *Humoral actions* may be slow and prolonged, or quick and short lived. For example, hormones have important effects on the ontogeny of reproductive behavior, migration and other seasonal activities. They control many rhythms, such as the sexual and menstrual cycles, and also exert a short-term influence on fear. All in all, multiple measures from freely behaving subjects over multiple time domains are necessary to specify

short- and long-term effects of brain neuropeptides and peripheral hormones.

4. There are many activities in which an animal could engage at any particular time: feeding, courtship, sleep, and so on. It is virtually impossible for an animal to carry out such different activities simultaneously, simply because the movements required are mutually incompatible. There are processes that determine which activity has priority at any particular time. Such processes are termed *decision processes* [33]. To unravel the process of deciding on different activities, an animal must be presented with alternatives and must be free to choose and experience the consequences of its own choice. Experimentally, this demands a focus on freely behaving subjects.

Here, we present general recommendations for applying optogenetics to the analysis of behavior in freely swimming zebrafish larvae. We offer data from a series of recent experiments in which a photoactivated adenylate cyclase (PAC) was used to manipulate the activity of pituitary cells in combination with newly developed assays for measuring goal-directed actions [34]. The pituitary is the major link between nervous and hormonal systems, which allow the brain to generate flexible behavior. Embedded in the hypothalamo-pituitary-adrenal (HPA) axis, pituitary corticotroph cells are known to control the release of glucocorticoids from the adrenal gland into the blood [35], yet their contribution to stress behavior had been difficult to pin down due to the limited accessibility of the hypothalamus and pituitary and the coupled release of hypothalamic and pituitary neuropeptides. The results of our experiments revealed rapid organizing effects of corticotroph cell products on locomotion, avoidance and arousal directly after the onset of stress. PAC contains a "blue light sensor using FAD" (BLUF) domain and, once activated by blue light, generates cAMP [36]. *Beggiatoa* PAC (bPAC) is an improved PAC, as compared to EuPAC [37, 38]. In zebrafish, bPAC had previously been used for analyses of stress reactions and neuronal repair [39, 40, 41, 42] (*see* also Chap. 4).

2 Materials

2.1 Common Buffers and Chemicals

1. E2 medium: 5 mM NaCl, 0.25 mM KCl, 0.5 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.66 mM $CaCl_2$, 0.71 mM $NaHCO_3$, pH adjusted to 7.0.
2. 0.2 mM 1-Phenyl-2-thiourea (PTU; Sigma-Aldrich, #P7629).

2.2 Generation of Transgenic Larvae Expressing Photo-Switchable Actuators (PSAs)

1. Wild-type zebrafish: e.g., cross between AB and TL strains.
2. Microinjector for DNA injection into single-cell-stage zebrafish embryos (e.g., Eppendof Femtojet).
3. Plasmid DNA allowing tissue-specific expression of PSAs in larvae.
4. Routine injection reagents: phenol red, injection mold or slide, glass capillary.
5. Fluorescence stereomicroscope to screen for embryos expressing PSAs according to signals from coupled fluorescence proteins (e.g., Leica MZ16F with excitation light source and filters).
6. Vibration-free incubator for raising larvae under controlled temperature and light conditions (e.g., RUMED type 3101).
7. Containers covered with light filters for raising embryos expressing PSAs; 550 nm long-pass filters (Thorlabs, Dachau, Germany) for bPAC.

2.3 Environmental Control

Several aspects of the environment can impinge on freely behaving larvae during a test, which makes it utterly important to control and monitor the global environment of the test chamber. The temperature, composition and motions of the medium as well as its overall level of illumination should be controllable. A straightforward approach consists of a cylindrical chamber equipped with a temperature monitoring sensor and an inlet, outlet and overflow, particularly if optogenetics is to be combined with proxies perfused into the medium and washed away in a highly controlled fashion. Figure 1 presents one such chamber:

1. The exemplary swimming chamber in Fig. 1 (internal diameter: 10 mm, height: 10 mm) has a transparent bottom and two opposite overtures, inlet and outlet (width: 2.5 mm, height: 400 μm). The chamber also had two cylindrical side channels (internal diameter: 400 μm) opposite to each other opening 200 μm above the transparent glass bottom, with their longest axis oriented at an angle of 30° relative to horizontal (**see** also **Note 1**).
2. A peristaltic pump allows the medium inside the chamber to circulate at a constant rate (IPC Ismatec, IDEX Health and Science GmbH, Wertheim, Germany).
3. A thermocouple (TS200, npi electronics GmbH, Tamm, Germany) monitors the temperature inside the chamber and provides feedback to a control system (PTC 20, npi electronics GmbH, Tamm, Germany; Exos-2V2 liquid cooling system, Koolance, Auburn, WA, USA).

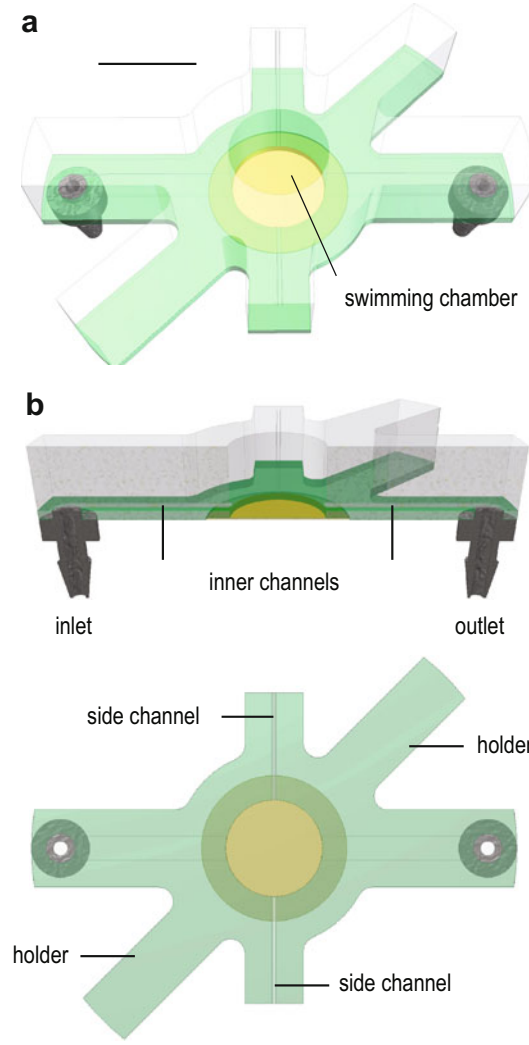

Fig. 1 Cylindrical swimming chamber with transparent bottom (**a**) and inner channels (**b**) allowing the medium to flow at constant or increasing temperature; side channels hold thermocouples constantly monitoring the temperature of the flowing medium. Scale bar, 10 mm. Modified from [34]

4. If experiments require perfusion, a computer-controlled perfusion system (Octaflow, ALA Scientific Instruments, Inc., Farmingdale, NY, USA) can be used to inject known solutions into a mixing compartment (internal diameter: 1 mm) situated 10 mm from the inlet of the swimming chamber. The mixing compartment can be connected to single reservoirs of solutions coupled to computer-controlled solenoid valves via Teflon tubing (internal diameter: 230 μm, outer diameter: 600 μm). TTL signals can trigger the opening and closing of the valves, allowing the solutions to be well mixed with the flowing medium before reaching the inner chamber.

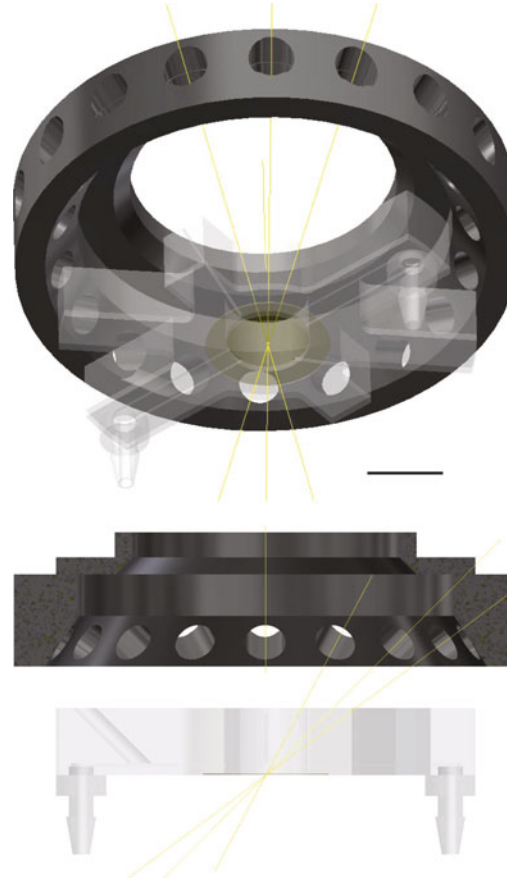

Fig. 2 LED ring on top of the test chamber in Fig. 1 for *blue* and *yellow light* stimulation in combination with video tracking. Scale bar, 10 mm. Modified from refs. 44, 45

2.4 Light Delivery

For the purpose of video recording freely behaving larvae, the investigator must adopt an approach for delivering light that is capable of providing homogeneous illumination within a relatively large chamber, particularly during tests of exploratory activity and goal-directed actions. Figure 2 presents an example based on a ring of LEDs.

1. The incident angle of the LEDs in Fig. 2, each pointing towards the center of the bottom surface of the chamber, allows for a homogeneous illumination of the chamber's inner compartment. Both the wavelength and intensity of the light needed for activating PSAs need to be separately adjustable and empirically determined for each transgenic line. This can be achieved by using distinct LEDs in combination with a control system for light delivery, composed of custom-made drivers for setting the light power, pulse generators for setting

the frequency and duration of a squared pulse of light, and a TTL control box for computer control of the LEDs.

2. A hand-held light power meter (Newport Corp., Irvine, CA, USA) can be used to measure light power (*see* **Note 2**)

2.5 Video-Tracking

Video tracking provides a suitable solution in many situations where a camera can access an entire swimming chamber from above. The essential features of a video-tracking system consist of a solid-state digital camera with a charge-coupled device (CCD) detector and a lens placed above the chamber so that the entire area of interest is in the field of view. A challenge arises from the poor contrast of the semi-transparent larva. Low contrast results in very small differences from background, which are difficult to track. The best solution is to equip the swimming chamber with a translucent bottom surface and provide homogeneous white and IR light from below through an array of LEDs covered by diffusing glass. With such an array, problems arising from object reflectance close to background brightness can be reduced if the LEDs have a wide range of brightness levels. A basic set of elements is as follows:

1. A light-proof enclosure for the entire set up placed on a vibration-free platform (Newport Corp., Irvine, CA, USA).
2. Infrared-sensitive cameras (25 frames*s^{-1}, ICD-49E B/W, Ikegami Tsushinki Co., Ltd. Japan, and 100 frames*s^{-1}, Firewire Camera, Noldus Information Technology, Wageningen, Netherlands) and lens (TV Lens, Computer VARI FOCAL H3Z4512 CS-IR, CBC; Commak, NY, USA) with a maximum 3× zoom (*see* also **Note 3**).
3. A source of infrared (IR) illumination opposite to the camera lens—we use a custom-made array of IR-LEDs below the swimming chamber of transparent bottom.
4. We use EthoVision XT software (Noldus Information Technology, Wageningen, Netherlands) for online video-tracking, but *see* also **Note 4**.

3 Experimental Procedures

3.1 Obtaining Transgenic Zebrafish Larvae for Optogenetics (See Note 5)

1. Clone a construct harboring the protein of interest (PSA) under the control of a tissue-specific promoter (*see* Chap. 2) and couple the PSA with a fluorescent protein to aid in subsequent identification/maintenance of the line.
2. Set up mating crosses of wild-type zebrafish the day before and collect embryos after the light onset on the following day.
3. Inject the construct (approximately 50–100 pg) into single-cell-stage embryos in the presence of 0.05% phenol red. If the

construct was generated with the Tol2 kit [43], then incubate with 100 pg Tol2 transposase RNA for 10 min prior to the injection.

4. After injection, maintain embryos inside an incubator at 28 °C on a 12:12 h light-dark cycle. Raise a maximum of 60 larvae in a (10 cm) petri dish with 15 ml of egg water, check at 1 day post fertilization (dpf) and remove dead embryos. At 5 dpf, transfer the larvae to the fish facility and start feeding.
5. Once the fish reach sexual maturity, identify transgenic carriers ("founders") by inter-crossing injected and wild-type fish and screening the progenies for the expression of the marker gene.
6. For transgenic larvae expressing PAC, set up a cross of *Tg (POMC:bPAC-2A-tdTomato)* with wild-type (AB/TL) fish. On the following day, collect the fertilized embryos and place a maximum of 60 embryos into a container with 15 ml of E2 medium. (If the experiment requires PTU, then add 0.2 mM at this point). Cover the container with a light filter (*see* Sect. 2.2) and place it in a temperature-controlled incubator at 28 °C on a 12:12 h light-dark cycle. At 3 or 4 dpf, depending on experimental needs, screen for larvae that express tdTomato in the pituitary under fluorescence microscope. For 4 dpf larvae, tricaine methane sulfonate (MS222, 0.01 mg/ml) may be added before (5 min) the screening to sedate the larvae; 3 dpf larvae are less likely to move and can be screened without MS222. Keep tdTomato-positive vs. tdTomato-negative larvae separately inside the incubator under filtered light as described above (*see* **Notes 6** and 7).

3.2 Optogenetic Interpellation of Behavior

Larval zebrafish are highly sensitive to optic stimuli [44]. When briefly (~5 min) adapted to darkness, for example, they react to a sudden dark-to-light transition with a brief period of increased locomotion directly after the light onset, followed by reduced locomotion during the light period and increased locomotion after the light offset. Notably, in briefly dark-adapted larvae, we observed that a squared pulse of either blue or yellow light not only elicits locomotor reactions, but also increases whole-body cortisol in a graded fashion, depending on light power and exposure time (Fig. 3) [41]. By virtue of its complexity alone, the regulation of behavior is susceptible to stress. The above finding is relevant because it shows that a light change in itself can be stressful and has, for that reason, the capacity to alter the regulation of a behavioral scheme; future tests on intact larvae must control for such an effect. The simplest way to activate PSAs without causing stress is to modify the wavelength composition of light while maintaining the overall illumination of the test chamber unchanged. Still, the fact that light can be stressful also has an advantage. It can be used in a dual role to understand how distinct modulators influence behavior

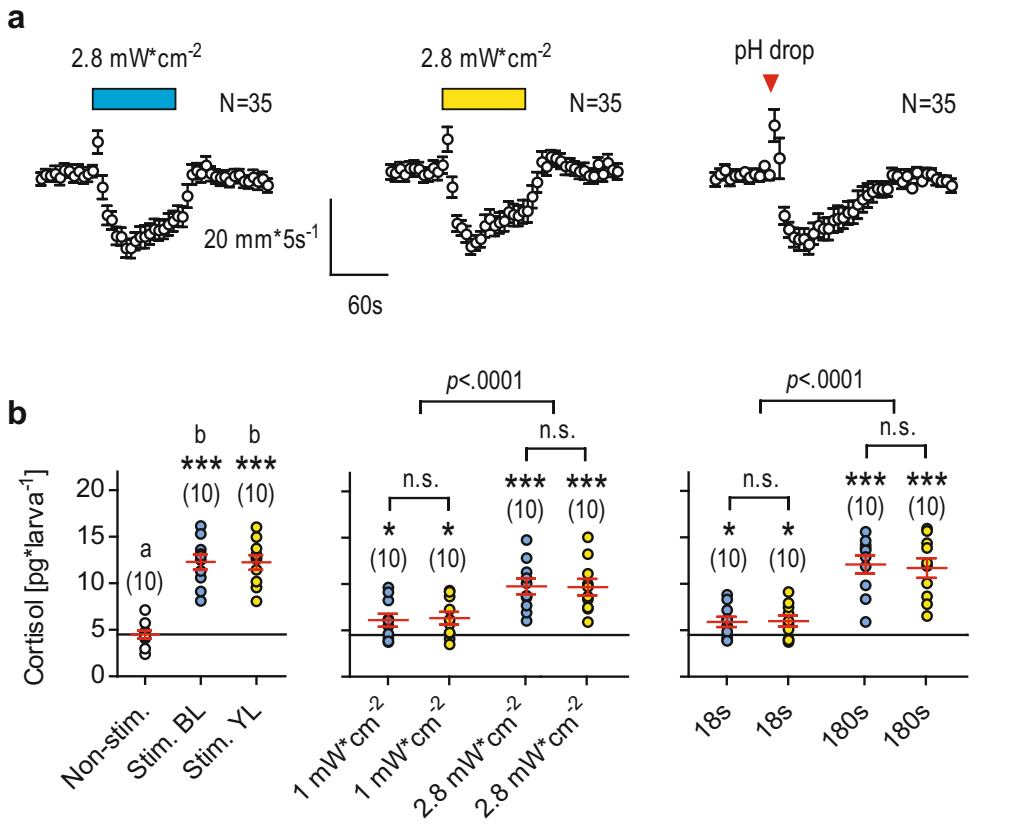

Fig. 3 (**a**) Left, Center, briefly dark-adapted larvae react to a squared pulse of either *blue* (left) or *yellow* (center) light with reduced and increased locomotion after the onset and offset of light, respectively. Right, they react similarly to a pH drop, a known potent stressor in fish. Locomotion measured as swim velocity [mm/(5 s)$^{-1}$]. (**b**) A squared pulse of light can increase whole-body cortisol (left, light power: 4.4 mW/cm^{-2}), depending on light power (center) and exposure time (right, light power: 2.8 mW/cm^{-2}). Left, One-Way ANOVA, $F(2,29) = 42.1$, $p < 0.0001$, followed by Bonferroni's tests. Center, Two-Way ANOVA, Wavelength factor: $F(1,36) = 0.01$, $p = 0.93$, Light Power factor: $F(1,36) = 19.8$, $p < 0.0001$, Wavelength × Light Power factor: $F(1,36) = 0.03$, $p = 0.86$, followed by post hoc comparisons. Right, Two-Way ANOVA, Wavelength factor: $F(1,36) = 0.03$, $p = 0.87$, Length factor: $F(1,36) = 52.1$, $p < 0.0001$, Wavelength × Length factor: $F(1,36) = 0.08$, $p = 0.79$, followed by post hoc comparisons. *$p < 0.05$, ***$p < 0.001$ after one sample t-tests against basal cortisol in nonstimulated larvae (*black line*), Non-stim: $t(9) = 0.01$, $p = 0.99$, Stim. BL: $t(9) = 9.6$, $p < 0.0001$, Stim. YL: $t(9) = 9.9$, $p < 0.0001$, 1 mW/cm^{-2} BL: $t(9) = 8.8$, $p < 0.0001$, 1 mW/cm^{-2} YL: $t(9) = 9.3$, $p < 0.0001$, 2.8 mW/cm^{-2} BL: $t(9) = 11.4$, $p < 0.0001$, 2.8 mW/cm^{-2} YL: $t(9) = 10.9$, $p < 0.0001$, 18 s BL, $t(9) = 2.4$, $p = 0.04$, 18 s YL, $t(9) = 2.5$, $p = 0.04$, 180 s BL, $t(9) = 7.7$, $p < 0.0001$, 180 s YL, $t(9) = 6.8$, $p < 0.0001$). Sample size in parentheses. See also De Marco et al. [34]

after the onset of stress. This is exactly what we did to study the role of pituitary corticotroph cells in mediating reversible phenotypic adaptations [34]. In larvae expressing bPAC specifically in pituitary corticotrophs, we showed that blue light can be used as both a potent stressor and a means to enhance corticotroph cell activity directly after the onset of stress, thus prompting locomotion

changes, meaningful adjustments in avoidance behaviors, higher levels of whole-body cortisol, and greater arousal. General recommendations from these experiments are listed below (*see* also **Note 8**).

1. Carry out individual video-recordings (i.e., one larva at the time), with different experimental groups intermixed throughout the day.
2. Provide each larva with an adaptation period of no less than 10 min to the test conditions.
3. Use an image spatial resolution of -no less than- 37 or 16 pixels per mm at 100 or 25 Hz, respectively.
4. Systematically use baseline recordings and compare baseline and test data across clutches, developmental stages, individuals and time of the day.
5. If experiments require drug substances using perfusion, use a maximum flowing medium of 200 $\mu l/min^{-1}$ in order to avoid rheotaxis.
6. Confirm that larvae do not show bias swim directions under IR light if experiments require darkness before and after the activation of PSAs.
7. Larvae show low response thresholds to light change. If light of a given frequency or light power is required during tests, locomotion profiles elicited by the selected frequency or power could mask, at least partially, changes related to PSA activation. Use light of different wavelengths as a means for wavelength-dependent optogenetic control of selective cell activity.

4 Notes

1. The level of baseline activity expressed by a larva is very important to consider when designing a swimming chamber. Larvae that move very little in a novel environment may cover only a small area of the chamber, undermining tests by inactivity. The shape and dimensions of the chamber will depend directly upon the type of targeted actions. A test for rheotaxis, e.g., will demand an elongated chamber as well as accurate control of the medium flowing through it. A test for feeding or any other goal-directed action causing a differential usage of space will demand a compartmentalized chamber so that a heterogeneous distribution of prey—or any other goal or condition—can be created (e.g., [34, 45]). Measuring escape reactions demands absent corners so that sustained displacements can occur.

2. Many light sources have a manual dial for setting the current intensity that determine light power, but this is not more than a rough guide to setting a desired power of light. The investigator needs to monitor the light power directly at the tip of the objective or at the bottom of the swimming chamber. The best practice is to determine the actual power of light that can reach a freely behaving larva by monitoring light power at specific wavelengths. For this purpose, place the sensor of the light power meter below a swimming chamber with a fully transparent bottom and measure light power—with and without liquid medium inside the chamber—across currents and distances from the light source.
3. If the lens is X mm above the bottom surface of the chamber and the width of the chamber is Y mm, the tangent of half the angle of view equals (Y/2)/X. The required focal length of a lens that can see the entire chamber must be less than or equal to (X × CCD)/(Y + CCD). The format of the CCD chip is important because the size of the chip and the focal length determine the angle of view. A CCD sensitive to very long wavelength or infrared light (IR) is desirable if optogenetic intervention is to be combined with behavioral recordings carried out in darkness.
4. Goal-directed actions by freely behaving larvae have already been measured using different video-tracking approaches [45, 46]. Open source software has also been published [47]. Commercially available systems provide readily measures of path length, latency until the subject enters specific zones, time and distance travelled in each such zone, number of entries, and so on, with the possibility to organize data on the basis of an overall estimate of mobility level. Usually, these systems locate the larva being tracked using background subtraction. An image is first stored before the larva is introduced into the chamber; it is important that illumination during this step be identical to that used when the larva is present. Once the larva is present, every few milliseconds—depending on the temporal resolution of the camera—another image is saved, and the difference between the new image and the background is determined for every pixel array. The entire difference array of Nx by Ny pixels is scanned and, for pixels where the absolute difference exceeds some preset threshold, the X and Y values for that pixel are added to two counters (Σx, Σy) and the pixel counters (nx, ny) are incremented by one unit. After the scan is complete, the coordinates of the weighted center of the image are found as Σx/nx and Σy/ny. A scale factor from an initial calibration of the image is finally used to convert pixels to millimeters and the vectors of scaled X and Y values for each image are used to determine path length and other measures.

Based on similar principles, algorithms to measure the overall motion level of not just one, but a group of larvae have also been implemented ([34, 45]). They can detect the movements of several larvae within one or several areas of interest using the pixel-by-pixel mean squared error (m.s.e.) of gray-scale transformed and adjusted images from consecutive video frames, given by m.s.e = 1 N Σpixel = 1 N (image frame, pixel–image frame−1, pixel)^2 where N corresponds to the total number of pixels of each frame. Notably, the m.s.e. index is sensitive to both the amount of motion and the number of swimming larvae, enabling estimates of differential space use or place preference in semi-compartmentalized chambers or in open chambers with varying nearby environments.

5. A list of different steps involved in obtaining transgenic larvae is given here for the sake of providing a complete overview involving the protocol. However, a detailed treatment of zebrafish transgenesis is well beyond the scope of this chapter. A general guideline for zebrafish husbandry and genetic methods can be found in "A Guide for the Laboratory Use of Zebrafish Danio (Brachydanio) rerio" by M. Westerfield, 5th Edition. For detailed instruction on microinjection in zebrafish, see published protocols [48, 49].
6. In studies involving noninvasive optogenetics and freely-swimming larvae, test groups are commonly identified by evaluating the expression of fluorescent proteins. This can be done either before or after the tests. Doing it before the tests aids in adjusting the size of the samples, which is particularly beneficial when the complexity of the tests makes them difficult to carry out and time consuming. By contrast, a blind design (i.e., doing it after the test) may prevent accurate control of the sample size, but it will rule out any possible bias during testing arising from the experimenter's hand. Sometimes a blind design is necessary for another reason: fluorescence proteins cannot be detected before a certain stage of development. Under such circumstances, screening for false negatives at one or more time points after the tests become utterly important.
7. Exposing transgenic larvae expressing optogenetic proteins to excitation lights during screening can activate the protein. New optogenetic tools with improved light sensitivity are activated at extremely low light and some could be activated even at ambient light [50] (*see* Chaps. 3 and 6). Light exposure before tests can therefore transform experimental outcomes. Transgenic larvae can be evaluated post hoc based on fluorescence.
8. *Do's*: Keep checking if medium temperatures in the testing chamber are constant throughout the whole testing period at

any day. Keep track of the exact time of the onset of light in your data. This is your fixed time point throughout all following data analysis. Depending on the data analysis planned, EthoVision XT allows for separate adjustments for the raw data output to Excel (amounts of data tend to be huge and difficult to compute sometimes). Create one master template per testing protocol each, and adjust specifics on a day-to-day basis in copies accordingly. *Don'ts*: Don't stick to the same recording order of different groups every day. Don't keep supplying tubes and the testing chamber filled with stagnant medium over longer periods of time (more than a few days) to prevent algae from building up and contaminating the system.

5 Outlook

The zebrafish provides a powerful system for optogenetics. In combination with high-resolution in vivo imaging, application of optogenetics in tethered larvae has already provided a great deal of insight to link neural circuit activity to principles of basic behavioral control. The next frontier for zebrafish optogenetics will be the analysis of complex behavior, which rests on joint work of four regulatory components such as internal states, motivations, adaptive responses to the environment, and decision processes. Such complex behavior cannot be accurately carried out employing restrained subjects, and thus the future zebrafish optogenetics research will rest increasingly on combination of precise and sophisticated genetic manipulations combined with exquisite and fine-grained analysis of behavior in freely moving subjects.

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