CHAPTER 3

Behavioral Screening Assays in Zebrafish

Michael B. Orger,[*] Ethan Gahtan,[*,‡] Akira Muto,[*] Patrick Page-McCaw,[*,†] Matthew C. Smear,[*] and Herwig Baier[*]

[*]Program in Neuroscience
Department of Physiology
University of California, San Francisco
San Francisco, California 94143

[†]Department of Biology
Rennselaer Polytechnic Institute
Troy, New York 12180

[‡]Department of Psychology
University of Massachusetts, Amherst
Amherst, Massachusetts 01003

I. Introduction

All animals show innate behaviors, which depend on the correct development and function of their nervous systems. The genes each individual inherits specify how the brain develops and operates and the way in which experience affects these processes. The search for the genetic underpinnings of behavior is rapidly

METHODS IN CELL BIOLOGY, VOL. 77

0091-679X/04 $35.00

expanding. Systematic screens for behavioral mutants provide an unbiased method to find the underlying genes (Benzer, 1973). In this review, we describe some of the behavioral screening assays we have devised in our laboratory to isolate mutations affecting the zebrafish nervous system. Zebrafish are ideally suited for a behavioral genetic approach (Guo, 2004; Li, 2001; Neuhauss, 2003). The larvae show a wide range of interesting behaviors, yet are small and can be produced in large numbers. Importantly, larvae do not need to be fed to survive until 8 days postfertilization (dpf.), and all the assays described in this review can be carried out in this period. Therefore, millions of fish can be tested in a large-scale screen while requiring relatively little maintenance. Adult behaviors can also be used for screens, on a smaller scale. Dozens of larval and adult behaviors have been described. Table I shows a small selection for which screens have been carried out or proposed in zebrafish.

What can we discover by using a behavioral genetic approach? First, such an approach is uniquely suited to discover the genes involved in the proper execution of behavioral programs, that is, in the acute function of the nervous system. Second, behavior provides a sensitive readout of developmental disruptions, many of which might be too subtle to be picked up by anatomical or histological screens. Thus, we can expect to identify factors required for neural cell fate decisions, differentiation, axon guidance, and synapse formation. Third, mutants obtained in a behavioral screen provide an alternative lesioning technique, complementary to surgical ablations but with different temporal and spatial resolution (Gahtan and Baier, 2004). Therefore, mutations provide unique insights into the function of neural circuits by identifying their essential components.

Well-designed behavioral screens are focused to find mutations specific to a particular neural system. Yet behavior is the endpoint of neural processing often involving tens or hundreds of cell types in a neural circuit or pathway, and therefore mutations might exert their effects at many places in the brain. This breadth should be considered an advantage of a behavioral genetic approach, because it allows the researcher to investigate a neural system in its entirety. This tight functional focus combined with a systemwide view can reveal rare but highly significant links between brain and behavior that would probably escape discovery by any other means.

II. General Considerations

Behavioral screens require responses to be reliably evoked in the laboratory. Thus, not all behaviors are equally conducive to this approach. As a rule, when looking for recessive mutations, the wild-type response probability in a given screening assay should be higher than 90% to be significantly distinct from the 75% expected from Mendelian ratios in a mutant clutch. Otherwise, a large number of false positives will be identified. In our experience, screening out false positives is

Table I
Zebrafish Behaviors Used in Genetic Screens

Behavior	Age	Selected references	Screen references
Swimming/motility	>4 dpf	Budick and O'Malley, 2000; Liu and Westerfield, 1988; Saint-Amant and Drapeau, 1998	Granato *et al.*, 1996
Photoentrainment and circadian regulation of activity; sleep	>4 dpf	Cahill *et al.*, 1998; Hurd *et al.*, 1998; Zhdanova *et al.*, 2001	
Touch-evoked twitching/fast start	>27 hpf	Eaton *et al.*, 1977; Gahtan *et al.*, 2002; Liu and Fetcho, 1999; Liu *et al.*, 2003; Lorent *et al.*, 2001; O'Malley *et al.*, 1996; Ribera and Nüsslein-Volhard, 1998; Saint-Amant and Drapeau, 1998	Granato *et al.*, 1996
Auditory fast start (acoustical/ vibrational startle)	>5 dpf	Eaton *et al.*, 1977; Kimmel *et al.*, 1974	Bang *et al.*, 2002; Nicolson *et al.*, 1998
Optokinetic response (eye movements pursuing visual motion)	>73 hpf	Easter and Nicola, 1996; Kainz *et al.*, 2003; Rick *et al.*, 2000; Roeser and Baier, 2003	Brockerhoff *et al.*, 1995, 1997; Neuhauss *et al.*, 1999
Optomotor response (whole-body movements following visual motion)	>4 dpf	Bilotta, 2000; Krauss and Neumeyer, 2003; Maaswinkel and Li, 2003; Orger *et al.*, 2000, Roeser and Baier, 2003	Neuhauss *et al.*, 1999
Visually mediated background adaptation (dispersal and aggregation of pigment granules in the skin)	>5 dpf	Kay *et al.*, 2001	Kelsh *et al.*, 1996; Neuhauss *et al.*, 1999
Visually mediated escape	Adult	Dill, 1972, 1974; Li and Dowling, 2000a,b	Li and Dowling, 1997
Prey capture	>5 dpf	Borla *et al.*, 2002; Budick and O'Malley, 2000	
Addiction; drug responses	Adult	Gerlai *et al.*, 2000	Darland and Dowling, 2001; Lockwood *et al.*, 2004

the most time-consuming part of a behavioral screen, even for our most robust assays. It is therefore essential to optimize the screening assay, ideally by using wild-type fish in a small-scale mock screen. Time allotted to perfecting the assay under "screen" conditions will certainly be recouped as time saved in the screen.

Choosing the time of day to run a particular assay is very important. Circadian rhythms can affect many aspects of behavior, including visual sensitivity (Li and Dowling, 1998). The robustness of both the optomotor and optokinetic responses is significantly affected by circadian rhythms. A reduction in wild-type responsiveness of just a few percent can seriously impair a screening assay.

Once the screen has begun, retesting of putants (putative mutants), by setting up the same pair of carriers and reevaluating the behavioral phenotype of their offspring, is essential. In our hands, with the screening assays presented here, we find that three trials with consistent results are sufficient to weed out false positives and inconsistent phenotypes. The recovery rate in the subsequent generation is higher than 50% for most assays, a rate comparable to morphological screens.

Innate behaviors are generally more robust than learned responses and therefore better suited for genetic approaches. None of the associative learning paradigms known to us fulfills the 90% criterion stated previously. However, we found that nonassociative learning (habituation and sensitization) is genetically tractable in zebrafish, as shown later. A systematic genetic dissection of learning and nervous system plasticity will be an important research program for the future, and it is likely that zebrafish will be an attractive system for this approach.

III. Behavioral Assays

A. The Optomotor Response

Larval zebrafish show a rich repertoire of visual behaviors (Clark, 1981; for reviews see Li, 2001 and Neuhauss, 2003). These include the optomotor response (OMR), in which a whole-field moving stimulus evokes swimming in the direction of stimulus motion. This response allows the fish to eliminate unwanted self-motion and avoid being swept away by water currents (Rock and Smith, 1986). When presented with a strong stimulus, 6–7 dpf. larvae respond more than 90% of the time. When fish perform an OMR appropriately, they physically separate themselves from those that do not, making this an excellent assay for an automated large-scale genetic screen.

We have designed a setup for automated testing of the OMR (Fig. 1A) (Orger *et al.*, 2004). Visual stimuli are displayed on a flat-screen CRT monitor that faces upward. Larvae are placed in custom-built long and narrow plexiglass tanks, or racetracks. These essentially restrict the larvae to swimming in one dimension, along the length of the racetrack. Twelve racetracks can be placed side by side on the monitor, and each can hold a clutch of up to 50 larvae. The stimuli, which consist of moving sinusoidal gratings, are generated in Matlab, using the Psychophysics Toolbox extensions (Brainard, 1997; Pelli, 1997), which can be downloaded for free (http://psychtoolbox.org). The gamma function of the CRT is measured by using a Minolta LS-100 light meter and corrected using Matlab, so that pixel brightness is linearly related to pixel value in our movies.

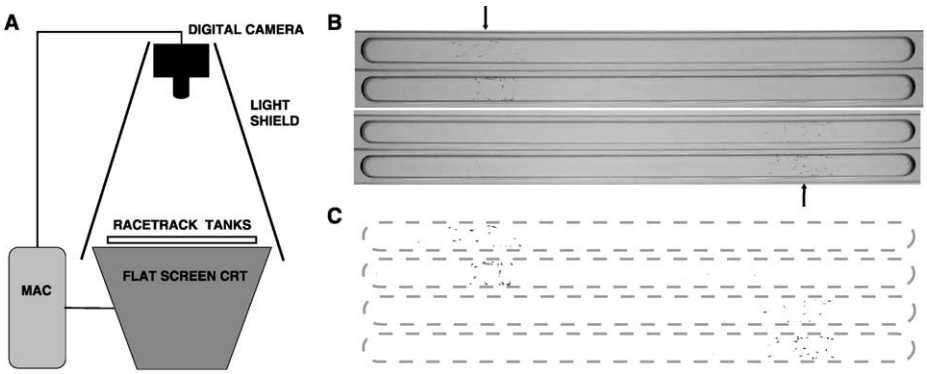

Fig. 1 The optomotor assay. (A) Schematic of the optomotor assay. Six- to seven-day-old fish larvae swim in shallow, elongated racetrack tanks on an upturned flat screen CRT monitor. A computer (Apple) controls stimulus presentation and a digital still camera (Nikon). External light is excluded. (B) Racetrack tanks pictured from above following stimuli consisting of converging sinusoidal gratings. In the upper image the grating converges at a point under the left half of the tanks (upper arrow). In the lower image, the convergence point is under the right half of the tanks (lower arrow). (C) Results of subtracting the images in (B) from each other and thresholding the resulting images. The outlines of the tanks disappear, and their position is indicated by the dotted line. The fish can be clearly seen clustered around the stimulus convergence points.

A digital camera (Nikon CoolPix) suspended above the monitor captures an image of the racetracks before and after each stimulus (Fig. 1B). Matlab can trigger the camera remotely by using a set of serial commands. These images are downloaded from the camera offline and analyzed by using custom macros in Object-Image (http://simon.bio.uva.n1/object-image.html). After subtracting two consecutive images to remove the background, the position of each fish is determined by using the "analyze particles" function of Object-Image (Fig. 1C). The average position of the fish in each tank before a stimulus is then subtracted from the average position after the stimulus. This gives the average distance swum by all the fish, which we call the optomotor index (OMI). Using this method, it is possible to screen thousands of clutches for recessive mutations affecting the OMR. However, because individual fish are not being tracked, it does not identify individual mutant fish. To sort mutants for further characterization and mapping, we use the following simple method. We play a movie in the leftward direction and move nonresponders into a new racetrack with a transfer pipette. After 30 sec the movie reverses, and the process is repeated. Even mutants with subtle defects can be efficiently sorted by using repeated iterations of this technique.

What mutants can we find with the OMR assay? First and most obviously, blind mutants are unable to see the stimulus. For example, mutants lacking photoreceptors or retinal ganglion cells have no OMR (Neuhauss *et al.*, 1999). A second class of mutants has motor deficits that impede the mutants' swimming ability. Finally, segregation of function is a common organizational feature of visual systems (e.g., Simpson, 1984), and so the most interesting class of mutations

could specifically affect the OMR while leaving other visually mediated behaviors intact. Finding such a mutation would help elucidate the neural circuit that mediates the OMR and the genes necessary for its development and function.

Computer-generated stimuli provide versatility because they can easily and rapidly be varied in several parameters, allowing us to ask more specific questions about the visual system. For example, we use cone-isolating stimuli in a motion-nulling paradigm to study color vision. Another study has focused on the acuity of the optomotor response by systematically varying the spatial frequency of the stimulus. By alternating stimuli, we can screen for mutants that respond to low but not high spatial frequencies.Acuity mutants can help us understand the limiting factors for spatial resolution in the visual system, such as the organization and grain of photoreceptor mosaics or the accuracy of retinotopy.

B. The Optokinetic Response

The optokinetic response (OKR) is a reflexive response, in which the eyes move to follow a large-field motion stimulus. In zebrafish, the OKR develops a few days after fertilization (Easter and Nicola, 1996). In our experiments, we use 6–7 dpf. larvae, which exhibit a robust OKR.

For optokinetic stimulation, a drum with black-and-white vertical stripes on its internal wall can be rotated mechanically around the zebrafish larvae (Brockerhoff *et al.*, 1995; Neuhauss *et al.*, 1999). An alternative method, which we use, is to project a computer-generated visual stimulus onto the internal wall of a drum (Roeser *et al.*, 2003). The advantage of this method is that we can change the color, spatial frequency, contrast, speed, or any other condition of the stimulus at any time during the recording to study a specific aspect of vision.

We use a public domain program, Image-J (http://www.rsb.info.nih.gov/ij/), for both stimulus generation and processing of the captured image. Image-J is a Java version of the NIH Image application, programmed by the same author, Wayne Rasband.

The setup for the OKR assay is shown in Fig. 2A, B. An animation of a windmill pattern of sine-wave gratings is generated by a computer. It is projected from below onto a white paper wall inside a drum, using an LCD projector (InFocus LP550), where the windmill pattern becomes vertical stripes. A robust OKR is elicited by using sine-wave gratings with a spatial frequency of 20 degrees/cycle moving at 10 degrees/sec. To focus the image, a wide-angle conversion lens (Kenko VC-050Hi, Japan) equipped with a close-up lens (King CU+1, Japan) and a neutral density filter (Hoya, ND4, Japan) is placed in front of the projector. At the center of the drum (6 cm height, 5.6 cm inner diameter), the zebrafish larvae are immobilized in 2.5% methylcellulose in egg water (0.3 g Instant Ocean/liter water) with their dorsal side up in the inverted lid of a 3.5-cm-diameter petri dish, which is placed on a glass table. To shield the fish from the direct beam of the projector and also to obtain a high-contrast image of the larvae, a white diffuser is

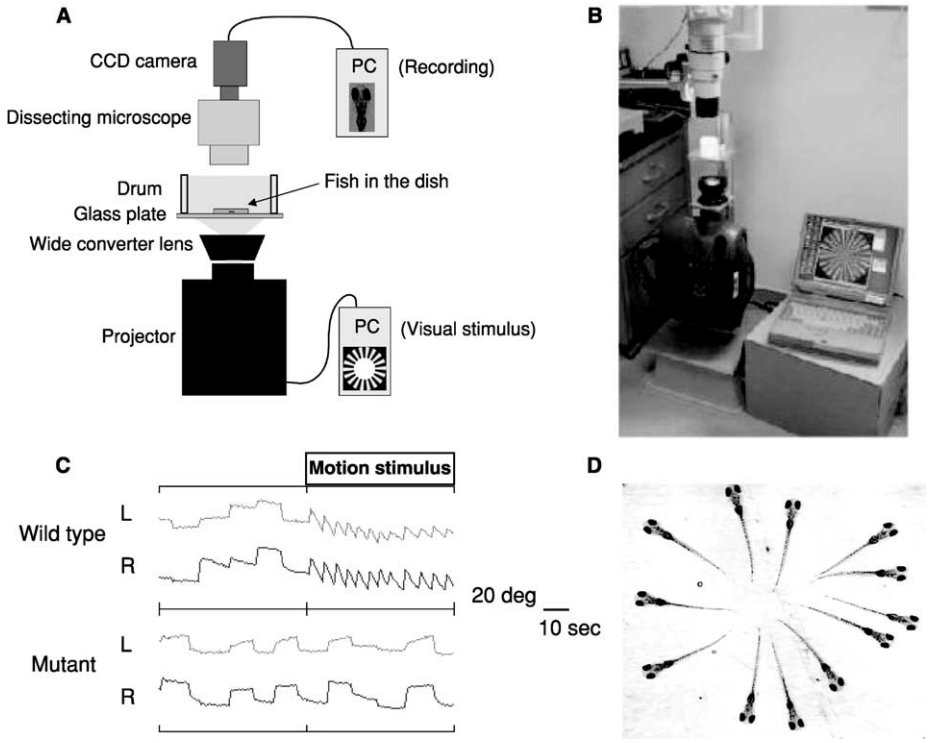

Fig. 2 The optokinetic assay. (A) A schematic of the optokinetic response (OKR) recording setup. An LCD projector projects computer-generated movies onto the inside of a drum, which surrounds a dish containing fish larvae. A CCD camera records movies of the larvae onto a computer, which analyzes their eye positions. (B) A picture of the OKR recording setup. A windmill grating stimulus can be seen on the computer screen. (C) OKR traces from a wild-type fish and a blind mutant fish at 7 dpf. Changes in eye positions of a wild-type larva and a blind mutant larva were plotted over time. Motion stimulus was presented during the second half of the recording period. L: left eye, R: right eye. Wild-type fish respond to the motion stimulus with alternating smooth tracking movements and fast reset saccades. (D) Image of 12 larvae in the recording chamber. Using such an arrangement, many larvae can be tested simultaneously.

placed below the dish. The fish are imaged by using a dissecting microscope (Nikon SMZ-800) with an arm stand and a CCD camera (Cohu MOD8215-1300).

Images are captured by a second computer through an LG-3 video capture board (Scion Corp.) at 2 frames/sec with Scion Java Package 1.0 for Image-J Windows (http://scioncorp.com). A custom Image-J plug-in identifies the eyes based on their dark pigmentation and then calculates their angle by using a standard algorithm (e.g., Seul *et al.*, 2002). The output is a plot of eye angle versus time. Figure 2C shows an example of eye position records of a wild-type and a blind mutant fish with and without an optokinetic stimulus.

Arranging larvae so that they are facing outward in a clock-face formation, as shown in Fig. 2D, enables us to record the eye movements of many fish simultaneously, allowing for high-throughput screening. We typically use batches of 12 fish. Wild-type response probability is close to 100%, and therefore screening 12 fish from a single clutch gives a high probability of detecting a recessive mutation.

C. Spontaneous Activity

An assay of larval spontaneous swimming activity can be used as both a primary screen assay and as an important control for other behavioral tests. Spontaneous locomotor movements in zebrafish develop through stereotyped stages, beginning at approximately 1 dpf (Budick and O'Malley, 2000; Saint-Amant and Drapeau, 1998). Variability among clutch mates is low enough to allow even small differences in activity, such as those caused by drugs (Zhdanova *et al.*, 2001), circadian cycles (Cahill *et al.*, 1998), or mutations (unpublished observations from our laboratory), to be detected. As a primary screening assay, spontaneous swimming activity is a blunt tool, because many different physiological defects could influence swimming. Therefore, we have found spontaneous activity most useful as a secondary test of locomotor function. For example, the OMR and prey capture assays are designed to test vision, but both depend on swimming ability, so a secondary spontaneous swimming test can help distinguish visual from motor defects.

The spontaneous activity assay described here can be run after larvae hatch and are freely swimming. We generally test only 7- to 8-day-old larvae with inflated swim bladders. Fish are tested in groups, with up to 6 fish in each rectangular well (3 cm × 7.5 cm) of a four-well clear acrylic plate (12.8 cm × 7.7 cm; Nunc, Roskilde, Denmark; Fig. 3A). The four-well plates are placed on a glass pane and imaged from below with a digital camcorder (Sony TRV-9). A light-diffusing white acrylic sheet is placed on top of the plates to produce a uniform light background in recorded images. Images are captured directly to the computer at a rate of 0.5 Hz, using the "time lapse" capture feature in Adobe Premiere. We generally record for 20 min, but longer durations or multiple sessions are possible. Recorded movies are analyzed by using Image-J. To be opened correctly in Image-J, the movies must first be saved without compression.

Image processing begins by subtracting each frame from the previous frame through the entire movie (Fig. 3B). The subtracted movie reveals only pixels that changed in value from one frame to the next; if a fish does not move in the 2-sec interframe interval, it will not be visible (dashed circle in Fig. 3A and B). The subtracted movie is thresholded at a level that includes the high-contrast fish but eliminates the background noise. A maximum projection of this movie provides a way to visualize the level of activity in each well at a glance (Fig. 3C). For a quantitative analysis, the "analyze particles" feature in Image-J is then used to count all fish in each well on each frame. The software is configured to count particles that are within a predetermined size range matching the size of the fish.

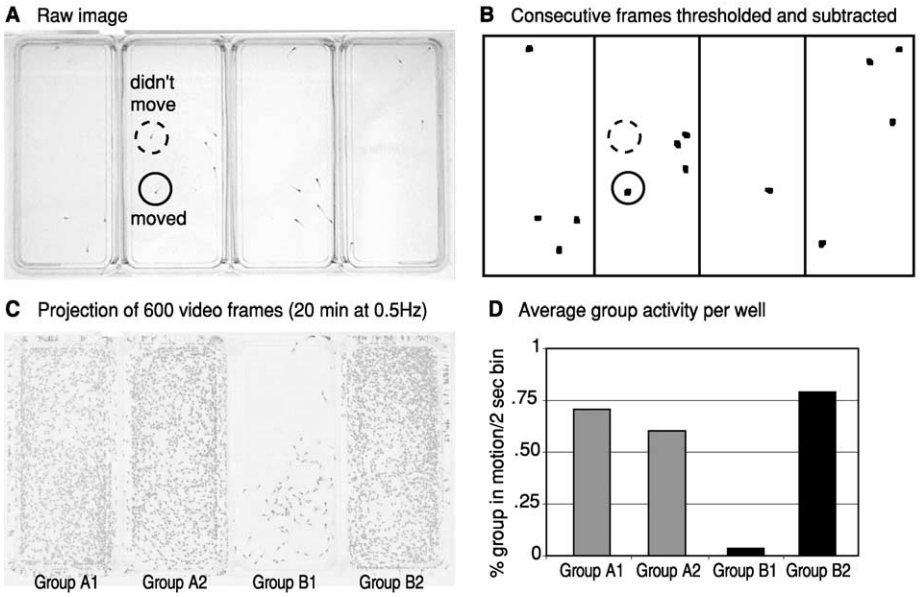

Fig. 3 The spontaneous activity assay. (A) Raw video frame. There are five fish in each well, which are visible as dark dots on the light background (e.g., inside the two circles). (B) An example of the successive frame subtraction procedure. This is the same image as in (A) after the previous image was subtracted from it. In this case, for clarity, the fish in the subtracted image were highlighted using the "Pixel Dilate" function in Image J. The dashed circle indicates a fish, visible in (A), which did not move and was subtracted out, and the solid circle indicates another fish that did move. (C) A projection of all 600 video frames after successive subtraction. Group B1 shows the fewest movement episodes. (D) Plot of the average number of movements per well across the 20-min observation period, divided by the number of fish. If every fish moved between every video frame, the value would be 1.

By keeping track of the number of fish in each well, we can determine the percentage of fish that have moved between any two frames. We quantify spontaneous activity as the average number of movements across all frames divided by number of fish in the well. Figure 3D shows the results of a single trial of two different OMR mutants and their siblings, whose projected tracks are shown in Fig. 3C. We test multiple fish per well, because we have observed in this assay and other behavioral assays that individually housed larvae are less active and responsive. To establish a statistically significant difference between two classes of fish, experiments need to be repeated multiple times.

D. Prey Capture

We have developed a prey capture assay as a test of fine visuomotor control (Fig. 4A). We have shown in control experiments in which fish feed in the light or the dark that feeding on paramecia is, to a large extent, visually mediated (Fig. 4B).

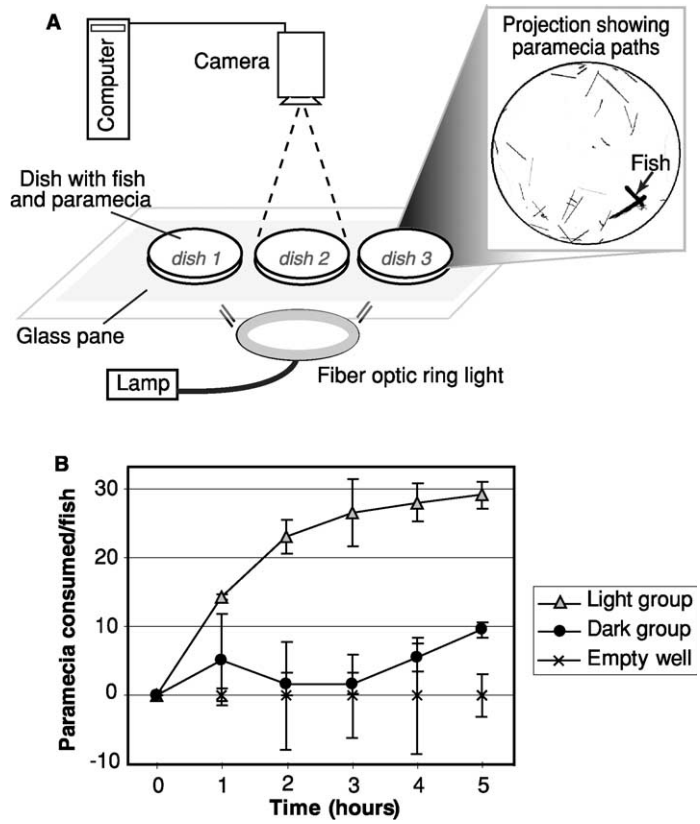

Fig. 4 The prey capture assay. (A) Schematic of the prey capture apparatus. Two hundred video frames are recorded for each dish (60 frames/sec), and paramecia are visualized as dark streaks on the projected image (inset). Sliding the glass pane facilitates recording multiple dishes in succession. (B) Fish kept in the dark during testing are impaired, suggesting a strong visual contribution to prey detection.

Our assay consists of tracking the number of paramecia in a dish over time as the larvae are feeding.

Live paramecia cultures and protozoa food pellets are obtained from Carolina Biological Supply Company (Burlington, NC). Cultures are grown in 500-ml plastic flasks at 28.5°C to a density of approximately 100 paramecia per milliliter, and new cultures are started every 2–4 weeks. Larvae are tested at 7–8 dpf., either individually in 3.5-cm petri dishes or in groups of up to four larvae in 5.5-cm petri dishes. Between 0.5 ml and 1.5 ml of paramecia culture is added to larvae dishes to achieve a ratio of about 50 paramecia per fish. Paramecia are also added to a dish containing E3 medium but no fish to determine the viability of paramecia over the 5-h assay period.

The equipment used is shown as a schematic in Fig. 4A. Dishes are placed on a glass pane illuminated from below with a fiber optic ring light to provide even illumination across the circular petri dish. Video images of the dish are recorded

with a high-speed digital camera (Redlake Motionscope PCI) positioned 30 cm above the dish. Two hundred video frames, captured at 60 Hz, are recorded for each dish, and dishes are recorded successively. Recording multiple dishes is facilitated by using a moveable glass base. An imaging-based counting method is used to determine the number of paramecia immediately after they are introduced (Time 0) and again hourly for 5 h. (The time course was determined by pilot studies.) Imaging-based counting is necessary because individual paramecia cannot reliably be seen in single still images from these recordings, but projecting multiple video frames allows each paramecium to be visualized as a dark streak across the background (Fig. 4A; paramecia can easily be distinguished from fish in the projected image).

Images are processed by using Image-J. First, the movies are projected by using the standard deviation *z*-projection method, which highlights changes in pixel values caused by the movement of paramecia or fish. Paramecia are marked and counted manually from the projected image, which is saved, whereas the larger movie file is deleted. Results are expressed as the number of paramecia consumed per fish. This number is then corrected for the spontaneous decline in paramecia over time, as determined by counts in the empty wells. In addition to spontaneous decline in paramecia number, there is also some variability in counting precision. In pilot experiments in which individual empty wells were counted repeatedly, we determined that the counting error was less than 5%. Analysis of variance can be used to determine whether treatment groups differ in prey capture performance. As in the spontaneous activity analysis, each treatment group should be run multiple times to determine trial-to-trial variability.

E. Startle Plasticity

Startle is a relatively simple reflex behavior that develops early, is homologous across species, and activates a motor response intended to facilitate escape from a threatening stimulus (Landis and Hunt, 1939). Startle can be elicited by multiple stimuli in different sensory modalities: in experimental systems, acoustic, visual, and tactile cues are most commonly used. The startle response is regulated by associative learning (e.g., fear conditioning), prepulse inhibition, and nonassociative learning (sensitization and habituation; Koch, 1999). Sensitization is the increase in response or response likelihood, and habituation is the decrease in response or response likelihood to a repeated stimulus. Defects in the regulation of startle have been observed in human diseases such as schizophrenia and Tourette's syndrome (Geyer *et al.*, 2001). Genetics are postulated to contribute to the etiologies of these defects. A screen for mutations that affect the regulation of startle will identify the genes, and ultimately the molecular and circuit mechanisms, that are required for experience-dependent plasticity in this system. In fish, startling stimuli elicit a specialized, highly stereotyped startle behavior called the fast start (Eaton and Hackett, 1984). The fast start consists of a large turn away from the stimulus followed by rapid swimming. The response latency (the time

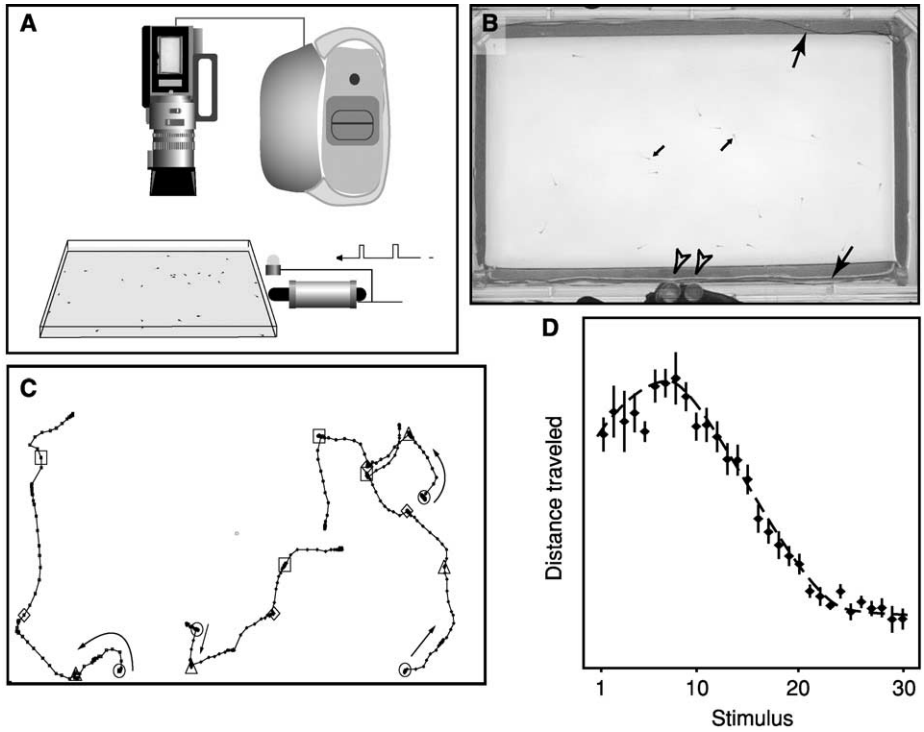

Fig. 5 The startle plasticity assay. (A) Diagram of the apparatus used to record the startle response. (B) One frame from a movie showing 7-day-old zebrafish larvae in the dish. Approximately 20 fish (small arrows) are placed into a rectangular dish illuminated from below. The dish-tap stimulus is presented by using a solenoid, and electrical shock stimuli (~1 VDC/cm) are presented by using a stainless steel electrode mesh running along the long sides of the dish (large arrows). All stimulus trains are controlled and timed by a G3 Macintosh using a custom AppleScript macro communicating over a serial bus to a custom-built stimulus controller. The stimulus controller also illuminates light-emitting diodes (arrowheads) in synchrony with the stimulus. Video of the behavior is captured by using a video camera, which streams the video data directly to a Macintosh computer for later processing. (C) Results of the tracking algorithm. Four fish are shown tracked across four stimulus responses. The position of the fish at each time point is indicated by the symbols, and the position of the fish at the time of the stimulus is indicated by the large symbols (circle, first stimulus; triangle, second stimulus; diamond, third stimulus; square, fourth stimulus; arrows indicate direction of motion). (D) Startle responses as a function of stimulus number as measured by mean distance traveled in response to a stimulus (±SEM, seven dishes of 20 fish). The response increases over the first few stimuli (sensitization) and then decreases (habituation).

between stimulus and response) is very short; it is among the fastest known motor responses. The turn, the rapid acceleration, and the short latency are all stereotyped.

High-speed videography (1000 frames/sec) of the fast start has been used for the detailed examination of the body kinematics during this behavior. (For a review, see Domenici and Blake, 1997.) The high temporal and spatial resolution of the data in these experiments come at the cost of allowing the study of only one startle

response in one animal per experiment. However, if the interest is in behavioral output rather than in kinematics, a much lower sampling rate and spatial resolution can be used. Using conventional video, we record the behavior of 20 fish simultaneously over 25 sec, extracting such startle parameters as the change in the animals heading, the velocity and acceleration during the response, the distance traveled and the duration of response.

Determining these parameters of a startle response for small groups of fish consists of four steps (Fig. 5A–D):

1. *Stimulus presentation*: The stimulus train is generated by a computer, allowing the presentation of multiple stimuli at precisely timed intervals. We typically use two different stimuli to elicit the startle response: a dish-tap and a mild electrical shock. The dish-tap stimulus is produced by a solenoid mounted so that the solenoid core taps the dish when activated. The electrical-shock stimulus is produced by stainless steel mesh electrodes running the length of the dish. Stimulus presentation at 2 Hz produces good nonassociative learning while keeping the video data relatively short (and the video files manageably small).

2. *Video capture*: To facilitate analysis of the motor behavior, video of the behavior is recorded from a Sony video camera directly to a computer hard drive. We determined that the camera's frame rate (deinterlaced to yield about 60 frames/sec) is sufficient to quantify startle behavior. The camera is set to record with short exposure times ($\leq$1/6000 sec) to ensure that the image is not blurred during high-speed swimming. Video capture is controlled through a VideoScript macro (http://www.videoscript.com).

3. *Movie processing*: The resulting videos of the startle responses are processed to remove the image background, allowing identification of each fish in each video frame. To remove background from the video images, an image of the dish with no fish in it is subtracted from each movie frame. Each frame is deinterlaced to extract the full temporal resolution of the movie. Background subtraction and deinterlacing are performed by macros written in VideoScript. Fish are then detected by using the "analyze particles" function of Object-Image; the x and y coordinates of each fish in each frame are recorded.

4. *Tracking*: Fish are tracked using the (x,y) positions supplied by Object-Image. Tracking allows the assignment of startle parameters to individual fish. The tracking algorithm attempts to find the nearest fish position in frame $i + 1$ to the track end found in frame i. Tracking and subsequent calculation of velocity, acceleration, heading, latency, and duration of response are made using macros written in Object-Image and MatLab (www.mathworks.com).

We use this assay to study nonassociative learning of the startle response. The startle-plasticity assay can be used to identify mutants that affect the parametric measures of startle, such as duration of response or distance traveled. We find that the distance traveled in response to a stimulus provides a reliable measure of the stimulus response. This assay can be used to find mutations that increase or

decrease this behavioral parameter, but because decreases in startle response might result from mutations that affect sensory or motor systems as well as integrative (i.e., learning) functions, we focus on mutants that have wild-type initial responses and show larger than wild-type response to later stimuli, because habituation is delayed. A screen for mutants with delayed habituation selects for the presence, rather than the absence, of a response, filtering out mutants with general sensory or motor deficits.

IV. Conclusions

We have described a set of assays of larval behavior in zebrafish. The setups are inexpensive, automated, and very adaptable, making them ideal as primary screening assays or for testing existing mutants in shelf screens. Although these assays cover a diverse set of behaviors, they do not come close to exhausting the range of behaviors shown by larvae. Moreover, by altering the stimuli, different types of screens can be conducted by using one assay. For example, we have described screening assays for mutants with altered stimulus specificity (Section III-A) and response plasticity (Section III-E). With so many possibilities, we can expect zebrafish to be a preferred model for behavioral screens in the years to come.

Acknowledgments

H. B. was supported by the grants from the following organizations: NIH RO1–EY12406, Packard Foundation, Klingenstein Foundation, and Sloan Foundation. Additional support was provided by NRSA (E.G.), NSF, UC Chancellor's Award, American Heart Association (M.C.S.), NARSAD (P.P-M.) Uehara, Naito (A.M.), and HHMI (M.B.O.). Thanks to Megan Carey for helpful comments on the manuscript.

References

Bang, P., Yelick, P., Malicki, J., and Sewell, W. (2002). High-throughput behavioral screening method for detecting auditory response defects in zebrafish. *J. Neurosci. Meth.* **118**(2), 177–187.

Benzer, S. (1973). Genetic dissection of behavior. *Sci. Am.* **229**(6), 24–37.

Bilotta, J. (2000). Effects of abnormal lighting on the development of zebrafish visual behavior. *Behav. Brain Res.* **116**(1), 81–87.

Borla, M. A., Palecek, B., Budick, S., and O'Malley, D. M. (2002). Prey capture by larval zebrafish: Evidence for fine axial motor control. *Brain Behav. Evol.* **60**(4), 207–229.

Brainard, D. H. (1997). The psychophysics toolbox, *Spatial Vision.* **10,** 433–436.

Brockerhoff, S. E., Hurley, J. B., Janssen-Bienhold, U., Neuhauss, S. C., Driever, W., and Dowling, J. E. (1995). A behavioral screen for isolating zebrafish mutants with visual system defects. *Proc. Natl. Acad. Sci. USA* **92**(23), 10545–10549.

Brockerhoff, S. E., Hurley, J. B., Niemi, G. A., and Dowling, J. E. (1997). A new form of inherited red-blindness identified in zebrafish. *J. Neurosci.* **17**(11), 4236–4242.

Budick, S. A., and O'Malley, D. M. (2000). Locomotor repertoire of the larval zebrafish: Swimming, turning and prey capture. *J. Exp. Biol.* **203**(17), 2569–2579.

Cahill, G. M., Hurd, M. W., and Batchelor, M. M. (1998). Circadian rhythmicity in the locomotor activity of larval zebrafish. *Neuroreport* **9,** 3445–3449.

Clark, D. T. (1981). "Visual Responses in Developing Zebrafish." University of Oregon Press, Eugene, OR.

Darland, T., and Dowling, J. E. (2001). Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proc. Natl. Acad. Sci. USA* **98**(20), 11691–11696.

Dill, L. M. (1972). Visual mechanism determining flight distance in zebra danios (*Brachydanio rerio*, Pisces). *Nature* **236,** 30–32.

Dill, L. M. (1974). The escape response of the zebra danio (*Brachydanio rerio*). II. The effect of experience. *Anim. Behav.* **22,** 723–730.

Domenici, P., and Blake, R. W. (1997). The kinematics and performance of fish fast-start swimming. *J. Exp. Biol.* **200,** 1165–1178.

Easter, S. S. Jr., and Nicola, G. N. (1996). The development of vision in the zebrafish (*Danio rerio*). *Dev. Biol.* **180**(2), 646–663.

Eaton, R. C., Farley, R. D., Kimmel, C. B., and Schabtach, E. (1977). Functional development in the Mauthner cell system of embryos and larvae of the zebrafish. *J. Neurobiol.* **8,** 151–172.

Eaton, R. C., and Hackett, J. T. (1984). The role of the Mauthner cell in fast-starts involving escape in teleost fishes. *In* "Neural Mechanisms of Startle Behavior" (R. C. Eaton, ed.), pp. 213–262. Plenum Press, New York.

Gahtan E., and Baier H. (2004). Of lasers, mutants, and see-through brains: Assigning cells and circuits to behaviors in zebrafish. *J. Neurobiol.* **59,** 147–161.

Gahtan, E., Sankrithi, N., Campos, J. B., and O'Malley, D. M. (2002). Evidence for a widespread brain stem escape network in larval zebrafish. *J. Neurophysiol.* **87**(1), 608–614.

Gerlai, R., Lahav, M., Guo, S., and Rosenthal, A. (2000). Drinks like a fish: Zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacol. Biochem. Behav.* **67**(4), 773–782.

Geyer, M. A., Krebs-Thomson, K., Braff, D. L, and Swerdlow, N. R. (2001). Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: A decade in review. *Psychopharmacology* **156,** 117–154.

Granato, M., van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J., and Nüsslein-Volhard, C. (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123,** 399–413.

Guo, S. (2004). Linking genes to brain, behavior, and neurological diseases: What can we learn from Zebrafish? *Genes Brain Behav.* **3,** 63–74.

Hurd, M. W., Debruyne, J., Straume, M., and Cahill, G. M. (1998). Circadian rhythms of locomotor activity in zebrafish. *Physiol. Behav.* **65,** 465–472.

Kainz, P. M., Adolph, A. R., Wong, K. Y., and Dowling, J. E. (2003). Lazy eyes zebrafish mutation affects Müller glial cells, compromising photoreceptor function and causing partial blindness. *J. Comp. Neurol.* **463**(3), 265–280.

Kay, J. N., Finger-Baier, K. C., Roeser, T., Staub, W., and Baier, H. (2001). Retinal ganglion cell genesis requires lakritz, a zebrafish atonal homolog. *Neuron* **30**(3), 725–736.

Kelsh, R. N., Brand, M., Jiang, Y. J., Heisenberg, C. P., Lin, S., Haffter, P., Odenthal, J., Mullins, M. C., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Kane, D. A., Warga, R. M., Beuchle, D., Vogelsang, L., and Nüsslein-Volhard, C. (1996). Zebrafish pigmentation mutations and the processes of neural crest development. *Development* **123,** 369–389.

Kimmel, C. B., Patterson, J., and Kimmel, R. J. (1974). The development and behavioral characteristics of the startle response in the zebra fish. *Dev. Psychobiol.* **7,** 47–60.

Koch, M. (1999). The neurobiology of startle. *Prog. Neurobiol.* **59**(2), 107–128.

Krauss, A., and Neumeyer, C. (2003). Wavelength dependence of the optomotor response in zebrafish (*Danio rerio*). *Vision Res.* **43**(11), 1275–1284.

Landis, C., and Hunt, W. A. (1939). "The Startle Pattern." Farrah and Rinehart, New York.

Li, L. (2001). Zebrafish mutants: Behavioral genetic studies of visual system defects. *Dev. Dyn.* **221**(4), 365–372.

Li, L., and Dowling, J. E. (1997). A dominant form of inherited retinal degeneration caused by a non-photoreceptor cell-specific mutation. *Proc. Natl. Acad. Sci. USA* **94,** 11645–11650.

Li, L., and Dowling, J. E. (1998). Zebrafish visual sensitivity is regulated by a circadian clock. *Vis. Neurosci.* **15**(5), 851–857.

Li, L., and Dowling, J. E. (2000a). Disruption of the olfactoretinal centrifugal pathway may relate to the visual system defect in night blindness b mutant zebrafish. *J. Neurosci.* **20**(5), 1883–1892.

Li, L., and Dowling, J. E. (2000b). Effects of dopamine depletion on visual sensitivity of zebrafish. *J. Neurosci.* **20**(5), 1893–1903.

Liu, K. S., and Fetcho, J. R. (1999). Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. *Neuron* **23**(2), 325–335.

Liu, D. W., and Westerfield, M. (1988). Function of identified motoneurones and co-ordination of primary and secondary motor systems during zebra fish swimming. *J. Physiol. Lond.* **403,** 73–89.

Liu, K. S., Gray, M., Otto, S. J., Fetcho, J. R., and Beattie, C. E. (2003). Mutations in deadly seven/notchla reveal developmental plasticity in the escape response circuit. *J. Neurosci.* **23**(22), 8159–8166.

Lockwood, B., Bjerke, S., Kobayashi, K., and Guo, S. (2004). Acute effects of a alcohol on larval zebrafish: A genetic system for large-scale screening. *Pharmacol. Biochem. Behav.* **77,** 647–654.

Lorent, K., Liu, K. S., Fetcho, J. R., and Granato, M. (2001). The zebrafish space cadet gene controls axonal pathfinding of neurons that modulate fast turning movements. *Development* **128**(11), 2131–2142.

Maaswinkel, H., and Li, L. (2003). Spatio-temporal frequency characteristics of the optomotor response in zebrafish. *Vision Res.* **43**(1), 21–30.

Neuhauss, S. C. (2003). Behavioral genetic approaches to visual system development and function in zebrafish. *J. Neurobiol.* **54**(1), 148–160.

Neuhauss, S. C., Biehlmaier, O., Seeliger, M. W., Das, T., Kohler, K., Harris, W. A., and Baier, H. (1999). Genetic disorders of vision revealed by a behavioral screen of 400 essential loci in zebrafish. *J. Neurosci.* **19,** 8603–8615.

Nicolson, T., Rüsch, A., Friedrich, R. W., Granato, M., Ruppersberg, J. P., and Nüsslein-Volhard, C. (1998). Genetic analysis of vertebrate sensory hair cell mechanosensation: The zebrafish circler mutants. *Neuron* **20,** 271–283.

O'Malley, D. M., Kao, Y. H., and Fetcho, J. R. (1996). Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. *Neuron* **17**(6), 1145–1155.

Orger, M. B., Smear, M. C., Anstis, S. M., and Baier, H. (2000). Perception of Fourier and non-Fourier motion by larval zebrafish. *Nat. Neurosci.* **3**(11), 1128–1133.

Pelli, D. G. (1997). The VideoToolbox software for visual psychophysics: Transforming numbers into movies. *Spatial Vision* **10,** 437–442.

Ribera, A. B., and Nüsslein-Volhard, C. (1998). Zebrafish touch-insensitive mutants reveal an essential role for the developmental regulation of sodium current. *J. Neurosci.* **18,** 9181–9191.

Rick, J. M., Horschke, I., and Neuhauss, S. C. (2000). Optokinetic behavior is reversed in achiasmatic mutant zebrafish larvae. *Curr. Biol.* **10**(10), 595–598.

Rock, I., and Smith, D. (1986). The optomotor response and induced motion of the self. *Perception* **15**(4), 497–502.

Roeser, T., and Baier, H. (2003). Visuomotor behaviors in larval zebrafish after GFP-guided laser ablation of the optic tectum. *J. Neurosci.* **23**(9), 3726–3734.

Saint-Amant, L., and Drapeau, P. (1998). Time course of the development of motor behaviors in the zebrafish embryo. *J. Neurobiol.* **37**(4), 622–632.

Seul, M., O'Gorman, L., and Michael, J. S. (2002). "Practical Algorithms for Image Analysis: Descriptions, Examples, and Code." Cambridge University Press, Cambridge.

Simpson, J. L. (1984). The accessory optic system. *Annu. Rev. Neurosci.* **7,** 13–41.

Zhdanova, I. V., Wang, S., Leclair, O. U., and Danilova, N. (2001). Melatonin promotes sleep-like behavior in zebrafish. *Brain Res.* **903**(12), 263–268.