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A novel high-throughput imaging system for automated analyses of avoidance behavior in zebrafish larvae

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Abstract

Early brain development can be influenced by numerous genetic and environmental factors, with long-lasting effects on brain function and behavior. The identification of these factors is facilitated by recent innovations in high-throughput screening. However, large-scale screening in whole organisms remains challenging, in particular when studying changes in brain function or behavior in vertebrate model systems. In this study, we present a novel imaging system for high-throughput analyses of behavior in zebrafish larvae. The three-camera system can image twelve multiwell plates simultaneously and is unique in its ability to provide local visual stimuli in the wells of a multiwell plate. The acquired images are converted into a series of coordinates, which characterize the location and orientation of the larvae. The developed imaging techniques were tested by measuring avoidance behaviors in seven-day-old zebrafish larvae. The system effectively quantified larval avoidance and revealed an increased edge preference in response to a blue or red ‘bouncing ball’ stimulus. Larvae also avoid a bouncing ball stimulus when it is counter-balanced with a stationary ball, but do not avoid blinking balls counter-balanced with a stationary ball. These results indicate that the seven-day-old larvae respond specifically to movement, rather than color, size, or local changes in light intensity. The imaging system and assays for measuring avoidance behavior may be used to screen for genetic and environmental factors that cause developmental brain disorders and for novel drugs that could prevent or treat these disorders.

1. Introduction

A multitude of genetic and environmental factors can affect human brain development and cause mental illnesses later in life. The identification of these factors is challenging, since such factors often act in complex signaling networks, during a limited window of time, and in specific regions of the developing brain. To better understand how genetic and environmental factors contribute to neural defects, high-throughput screening technologies have been developed [1]. Particularly promising are the ‘high-content’ screens, which allow for the simultaneous analysis of multiple parameters in a cellular environment [2–6]. High-

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content screens are typically carried out by imaging specific cell types in multiwell plates using automated microscopes. However, no single cell line can recapitulate the diverse neural cell types and neural connections that are present in the brain. A broader approach is to screen for brain defects by studying behavior in whole organisms. This behavioral approach is attractive, since it may reveal defects in multiple neuronal cell types in multiple regions of the brain. Moreover, behavioral assays can reveal defects not only in neural patterning, but also in neural function. For logistic reasons, behavioral assays are incompatible with high-throughput screening in most vertebrate species. Notable exceptions are mice, which can be screened for behavioral phenotypes using various automated assays [7, 8] and the zebrafish, *Danio rerio*.

Zebrafish are widely used to examine embryonic development and recently gained traction as a model system in behavioral neuroscience [9–15]. Adult zebrafish are used to study anxiety, learning, and social behaviors and there is much interest in the behavior of zebrafish larvae, since these larvae are ideally suited for large-scale studies. A modest zebrafish colony can produce hundreds to thousands of synchronously developing embryos on a daily basis. Zebrafish larvae hatch from their chorion at 2–3 dpf (days post fertilization) and display a range of swimming, hunting, escape, and avoidance behaviors during the first week of development [12]. The developing larvae are only a few millimeters long and can be imaged in 6, 12, 24, or 96-well plates. The size of the wells is likely to affect larval behavior, similar to tank-size dependent behaviors in adult zebrafish [16]. While the smaller wells of the 96-well plates are well-suited to screen for defects in larval activity [17, 18], the larger wells of the 6- and 12-well plates may be beneficial when imaging place preference, avoidance of local stimuli, or social behaviors [12]. Automated imaging systems have been developed for the analysis of behavior in zebrafish larvae, and these systems have been used in high-throughput screens for small molecules that alter behavior [18, 19]. Some of these imaging systems are now commercially available. For example, Noldus and ViewPoint have developed elegant automated imaging systems for the analysis of larval activity in a single multiwell plate.

In the present study, we describe the development of a novel imaging system for high-throughput analyses of behavior in zebrafish larvae. The three-camera system can image twelve multiwell plates simultaneously and is unique in its ability to provide local visual stimuli in the wells of a multiwell plate.

2. Materials and Methods

2.1 Zebrafish larvae

Adult wild type zebrafish were originally obtained from Carolina Biological and are maintained at Brown University as a genetically diverse outbred strain. The fish are kept in a mixed male and female population on a 14 hour light / 10 hour dark cycle and are fed a combination of flake food and frozen brine shrimp. Embryos were collected from the tanks at ‘dawn’ and were raised at 28.5°C in egg water, containing 60 mg/L sea salt (Instant Ocean) in deionized water and 0.25 mg/L methylene blue as a mold inhibitor. Embryos were grown at an approximate density of 250 embryos per liter in Aquatic Habitats 2-liter breeder tanks. Unfertilized eggs were removed from the breeder tanks at 1 day post fertilization (dpf). The embryos hatch from their chorion at 2–3 dpf. The free-swimming fish are referred to as ‘larvae’ from 3 – 29 dpf, ‘juvenile fish’ from 30 – 89 dpf, and ‘adults’ starting at 90 dpf, in line with the staging series on ZFIN (http://zfin.org/zf_info/zfbook/stages). It should be noted that the transition from larvae to juvenile fish is somewhat arbitrary, since all major organs have developed before this time.

2.2 The zebrafish imaging system

The zebrafish imaging system was built in a WoodCrafters tower cabinet, measuring $180 \times 40 \times 40$ cm (Fig. 1). The top shelf of the cabinet was replaced by a wooden shelf with a 14×16 cm hole and a bracket to mount the camera (supplement 5). For image acquisition, we chose a 15 megapixel Canon EOS Rebel T1i digital camera with an EF-S 55–250mm f/4.0–5.6 IS zoom lens (www.canon.com). The camera is connected to a continuous power supply (Canon ACK-E5 AC Adapter Kit) and a laptop computer (Acer Aspire 5517-1127). The camera is controlled with Canon's Remote Capture software, which is included with the camera. A second Acer Aspire 5517 laptop is used to provide visual stimuli to the larvae (see supplement 1 and 2 and Fig. 1C). The 15.6" LCD screen has a 1366×768 pixel resolution and a brightness of 220 cd/m^2 . To avoid moiré patterns in the images, a plastic diffuser (Pendaflex 52345) was placed between the LCD screen and the multiwell plates. A single imaging unit, capable of imaging 4 multiwell plates simultaneously, costs approximately \$2,500 in supplies, including the cabinet, mounting, the camera and lens, the AC adapter kit, a SanDisk 8GB SDHC memory card, two laptop computers, and Microsoft PowerPoint and Excel software. We built three adjacent units to simultaneously image 12 multiwell plates.

2.3 Imaging zebrafish larvae

Zebrafish larvae were imaged at 7 dpf at which point the larvae display a range of locomotor behaviors, but have not yet depleted their yolk sac. The larvae are 4 – 5 mm long at this time. Twenty minutes before the start of an imaging experiment, the larvae were transferred to 6-well plates (Corning Costar no. 3506). The wells contained an agarose ring to improve the optics at the edge of the swimming area [20]. The rings were made by pouring 5 ml of melted agarose (0.5% in deionized water) into each of the wells. Once solidified, we used a plastic vial to stamp a 27 mm diameter $\times$ 5 mm deep hole in the agarose. The 6-well plates containing zebrafish larvae were placed on the laptop's LCD screen and the lids of the plates were removed to avoid condensation. Canon's software has a 'Remote Shooting' feature for computer-controlled image acquisition in video or interval mode. For video acquisition, a single 6 well plate was imaged for 15 minutes at 30 frames per second (fps), using a 1280×720 pixel resolution. The video was first stored in a compressed 3.2 GB movie (.mov) format on the camera's memory card and was subsequently transferred to the computer's hard drive. In the interval mode, images can be acquired at higher resolution, which allows for the simultaneous imaging of 4 multiwell plates with one camera. In addition, the images were loaded directly into the computer, which made it possible to acquire data files that were much larger than the capacity of the camera's memory card. We used the following camera settings: image quality = 4752×3168 pixels, high-quality JPEG compression (6 MB / image), lamp = outdoor, iso-speed = 100, F = 5.0, and exposure time (T_v) = $1/20$ sec. The larvae were imaged for 1 hour, using either a 1 minute interval (60 images per hour), a 30 second interval (120 images per hour), or a 6 second interval between images (600 images per hour).

2.4 Visual stimuli

The visual stimuli are created in Microsoft PowerPoint, which allows investigators to quickly change parameters of the visual stimuli, such as color, size, or movement (supplement 1 and 2). We created blue and red discs with 1.35 cm diameters that were either stationary or bouncing from the left-to-the right, and back, in the upper half of the well (using 'custom animation' in PowerPoint). While these are actually flat two-dimensional colored discs, we will refer to them as 'bouncing balls' throughout the remainder of the paper. The bouncing balls travel from the left to the right in 2 seconds over a straight 2 cm trajectory. The colors of the blue and red balls were optimized for the image analysis, i.e. the RGB values were 0, 0, 250 for the blue balls and 255, 0, 0 for the red balls. With these

values, the bouncing balls disappeared in the red or blue channels when splitting the channels of a full color image (Fig 1). We also created two adjacent blinking balls in the upper half of the dish which turn ON or OFF every 2 seconds. The two blinking balls were set to turn ON simultaneously or on an alternating cycle.

2.5 Automated image analysis in ImageJ

To obtain quantitative information from the one-hour imaging experiments, we developed an ImageJ macro that automatically measured the location and orientation of zebrafish larvae in multiwell plates (Fig. 2). The macro can be downloaded from the supplementary information (supplement 3). The header of the macro provides information on how to install the software as a plugin in ImageJ. The macro generates a 'Results' file, which includes the image name, larval area in pixels, mean intensity, X, Y coordinates of the centroid, X, Y coordinates of the center of mass, X, Y, width, and height coordinates of the bounding box, well number, and X, Y coordinates of the midpoint of the well. This file can be opened in MS Excel for further analysis.

2.6 Analysis of the video files

We used QuickTime Pro to export the 15 minute videos at 5 frames per second. This export generated a sequence of 4500 TIF images (2.7 MB/image, 12 GB total). We then imported a sequence of 150 images ImageJ (version 1.43u), which corresponds to a 30 second segment of the video. The 150 images were collapsed as a minimum intensity projection (ImageJ, image, stacks, z-project). This analysis provided a detailed map of the swimming patterns during a 30 second window of time (Fig. 3). We also wrote an ImageJ macro to collapse large sets of images that exceed the available RAM of the computer (Supplement 4). This macro can be used not only for the video analysis, but also to obtain an overview of larval location and activity in a time lapse recording.

2.7 Data analysis in MS Excel

The location of the larvae in the well was calculated in MS Excel as described previously [20, 21]. In summary, the X,Y coordinates of a larva's centroid are compared to the midpoint of the well to determine if a larva is located up, down, left, or right in the well. For example, if the Y value of a larva's centroid is smaller than the Y value of well's midpoint, the larva is located 'up' in the well. If not, the larva is located 'down' in the well. This translates to the following equation in MS Excel: `=IF(G32<P32,"up","down")` with cell G32 containing the Y coordinate of the larva's centroid and cell P32 containing the Y coordinate of the well's midpoint. In the present study, we used the X, Y values of the larva's centroid and the midpoint of the bounding box to determine if a larva is facing up, down, left, or right ($\pm 45^\circ$). The parameters 'up or down', 'center or edge', and 'inward or outward' were all defined to have an expected 50:50 ratio in a random distribution.

2.8 Statistical Analyses

Differences between experimental groups were tested for significance using Microsoft Excel's two-tailed t-test with unequal variance. Unless specified otherwise, we compared the behavior of larvae exposed to visual stimuli vs. the behavior of larvae without visual stimuli. To assure that the measurements are independent, larvae exposed to visual stimuli were compared to control larvae without visual stimuli, on a per-well basis (n = number of wells).

3. Results

3.1 The zebrafish imaging system and automated image analysis

When designing the zebrafish imaging system, we aimed for a system that would be user friendly, readily duplicated for high-throughput studies, and effective in the automated analysis of behavior. To achieve the first two goals, the system was built from widely available components, including a tall cabinet, a high-resolution Canon camera for image acquisition, and a laptop computer to present PowerPoint slides to the larvae (Fig. 1 A–D). However, to be effective in the automated analysis of behavior, new protocols for image analysis had to be developed. Acquired images were analyzed in ImageJ, which can be downloaded free of charge from the NIH (<http://rsb.info.nih.gov/ij/>). The general approach was based on a previous method that identifies individual larvae using ImageJ's particle analysis [20, 21]. However, three issues had to be resolved for the current high-throughput imaging system; (a) the location of the larvae was obscured by the visual stimuli, (b) it was difficult to determine the orientation of larvae when imaging four multiwell plates simultaneously, and (c) large image files could not be opened and analyzed when the size of these files exceeded the computer's RAM.

- a. The first issue of interference by visual stimuli was resolved by separating the colors of the full-color images. For example, blue stimuli are visible in the red and green channels, but not in the blue channel of a color image (Fig 1E–H). Thus, the images were analyzed in the blue channel only, where blue stimuli are invisible. Similarly, when presenting red stimuli, we used the red channel for image analysis. Any remaining shadows were removed by background subtraction and small air bubbles or specks of dust were removed by a size filter in ImageJ's particle analysis (Fig I–K). With these techniques, 98.3% of the larvae were correctly detected (sem=0.7, n=12 wells, 1 larva per well, imaged for 20 minutes), indicating that this protocol can be used to effectively identify larvae in the presence of visual stimuli.
- b. To determine the orientation of the larvae, we previously used measurements of the larva's centroid and center of mass [20, 21]. The centroid is located toward the head and the center of mass is located toward the tail. However, when imaging four multiwell plates with a 15 megapixel camera, the larva's centroid and center of mass were just one pixel apart (avg=1.07 pixels, sem=0.03 n=237). To obtain a more accurate parameter of larval orientation, we also included measurements of a bounding box, the smallest straight rectangle that encloses a larva (Fig 1L). The midpoint of the bounding box is located in the tail, nearly 7 pixels away from the centroid (avg=6.54 pixels, sem=0.14, n=237). The automated analysis of larval orientation was evaluated using 202 images of larvae, in which we could unambiguously determine the orientation as up, down, left or right by manual scoring. We then measured the orientation of the same larvae by automated image analysis, using the bounding box method. In 98.5% of the cases, the orientation in the automated analysis matched the orientation in the manual analysis, indicating that the bounding box method is an efficient approach for the automated analysis of larval orientation.
- c. The third issue was that large image files could not be opened and analyzed when these files exceeded the computer's RAM. This problem was solved by writing an ImageJ macro that analyzes a sequence of images, one image at a time (Fig 2). Thus, when the analysis of one image is completed, the image is closed, before opening the next image. The macro makes it possible to automatically analyze hundreds or even thousands of high-resolution images in sequence. The developed

macro for automated analysis of zebrafish larvae in multiwell plates is included in the supplementary information of this manuscript (supplement 3).

Overall, the imaging system and image analysis techniques make it possible to automatically analyze larval behavior and to project local visual stimuli in multiple multiwell plates.

3.2 Optimization of imaging speed for avoidance behavior

The developed imaging system was tested by measuring avoidance behavior in zebrafish larvae at 7 days post fertilization (dpf). We used 6 well plates to give the larvae ample space to swim away from the visual stimuli. Larvae were exposed to a 'bouncing ball', a 1.35 cm disc that moved from the left to the right and back in the top half of the well (see supplement 1 and 2). The response of the larvae to the bouncing ball was examined by recording 15 minute videos at 30 frames per second. These short videos revealed complex patterns of resting, swimming, and turning at various velocities (Fig 3). The behaviors were often directed away from the bouncing ball towards the bottom edge of the well, although we also observed larvae swimming or resting in the area of the bouncing ball. To quantify these intriguing but variable behaviors, we aimed for longer recording times and a larger number of wells. However, in the 30 fps video mode, such an expansion would quickly result in hundreds of thousands of images, which are difficult to store and analyze due to time and file size constraints ($30 \text{ fps} \times 3600 \text{ sec/hr} = 108,000 \text{ images / hr}$). In an effort to standardize the experiments, minimize file size, and speed up the ImageJ analysis, we analyzed larval behaviors at 6, 12, 18, 24, 30, and 60 second intervals (Fig 4). We then examined the following parameters: (1) swim speed and file size, (2) the preference for the bottom half of the well, and (3) the preference for the edge of the well. (1) The measured swim speed and file size are both dependent on the rate of image acquisition (Fig 4A). A trend line of the swim speed, fitted by polynomial regression ($y = -0.0004x^3 + 0.0541x^2 - 2.5743x + 53.935$, $R^2=0.9997$), indicates that 75% of the swim speed is measured at a 6 second interval. A 6 second interval corresponds to 600 images per hour or 3.6 GB per hour (one compressed image = 6 MB). A typical one hour recording with 600 images can be analyzed in a few hours by automated image analysis in ImageJ. When switching to a 60 second interval between images, the measured swim speed is approximately 7 times lower than the actual swim speed. However, the storage requirements and analysis time may be reduced 10-fold. (2) The preference of the larvae for the bottom half of the well (away from the bouncing ball) was examined in a one hour recording (Fig 4B). The one hour recordings analyzed at 6 second intervals, showed that the larvae were located in the bottom half of the well 82.1 % of the time (sem= 5.6, n=11 wells with 1 larva per well). The results were nearly identical when analyzing the data using longer intervals between the images ($p=0.89$, 6 vs. 60 sec interval). (3) The preference for the edge was examined by dividing the well into inner and outer halves (Fig 4C). Both halves were matched for area. The one hour recordings analyzed at 6 second intervals revealed that larvae were located in the outer half of the well 88.4 % of the time (sem= 3.4, n=11 wells). Again, the results were nearly identical when analyzing the data using longer intervals between the images ($p=1.00$, 6 vs. 60 sec interval). Based on these results, we conclude that the swim speed is most accurately measured when imaging at a 6 second, or shorter, interval. However, the location of the larvae can be accurately measured using a 60 second interval, which saves storage space and speeds up the image analysis. Thus, high-speed imaging may be the preferred approach for measuring larval activity and low-speed imaging may be beneficial for high-throughput analyses of avoidance behavior.

3.3 The one-fish bouncing ball assay

To obtain quantitative information on larval avoidance behavior, we examined larval swimming patterns in a larger number of wells using a 60 second interval between images

(Fig. 5). We found that larvae exposed to the bouncing ball spent more time down in the bottom half of the well away from the bouncing ball (Fig. 5A), i.e. the percentage of time spent in the bottom half of the well increased significantly from 56.4% (s.e.m.=2.0, n=119) without visual stimuli to 67.2% (sem=2.4, n=82) in the presence of a blue bouncing ball ($p<0.001$). Larvae exposed to the bouncing ball also spent significantly more time at the edge of the well (avg=87%, sem=1.0), than larvae without visual stimuli (avg=76%, sem=1.4, $p<0.0001$), Fig. 5B,C. In addition, larvae exposed to the bouncing ball displayed an increased outward orientation (avg=70.9%, sem=1.4), compared to the larvae without visual stimuli (avg=65.9%, sem=1.0, $p<0.01$), Fig 5D. We did not observe a statistical difference in swim speed between the control larvae (avg=9.2 mm/min, sem=0.33) and the larvae exposed to the bouncing ball (avg=9.9 mm/min, sem=0.47), Fig 5E. However, larvae exposed to the bouncing ball move significantly faster when they are in the upper half of the well (avg=12.6 mm/min, sem=0.56) than they do down in the lower half of the well (avg=10.0 mm/min, sem=0.40, $p<0.0001$). Similarly, we did not observe a statistical difference between the percent rest in the control larvae (avg=36%, sem=2.2) and the larvae exposed to a bouncing ball (avg=34%, sem=2.2), Fig 5F. However, larvae exposed to a bouncing ball rest more frequently down in the dish (avg=32%, sem=2.3) than they do up in the dish (avg=23%, sem=2.5, $p<0.05$). Since larvae are more likely to be immobile in the bottom half of the well, this immobility is likely a resting behavior, rather than a freezing behavior. Overall, the obtained results indicate that avoidance behavior can be accurately measured in multiwell plates. A potential obstacle for high-throughput studies is the number of wells that need to be imaged in order to obtain statistically significant results. For example when studying thousands of genes or small molecules, it would be desirable to image no more than 12 wells per experimental group. However, the avoidance behaviors of individual larvae were highly variable and the 12 wells proved to be insufficient for measuring a significant avoidance response. We conclude that the one-fish bouncing ball assay can be used to accurately measure avoidance behaviors, but that the assay would need to be improved in order to be suitable for high-throughput studies.

3.4 The two-fish bouncing ball assay

The two-fish assay was developed in part to examine larval interactions, and in part to improve the statistics of the avoidance response. The experimental setup and visual stimuli are the same as described above for the one-fish assay, but we added two larvae per well, instead of one. Since the two larvae may interact, the analysis was carried out on a per-well basis, rather than a per-larva basis (assuring that the measurements are independent). The larvae were imaged without visual stimuli (n=93 wells) or in the presence of a blue bouncing ball (n=54 wells). We found that larvae exposed to the bouncing ball spent significantly more time down in the dish (avg=65%, sem=1.7), than larvae without visual stimuli (avg=55%, sem=1.5, $p<0.0001$), Fig 6A. Larvae exposed to the bouncing ball also spent significantly more time at the edge of the well (avg=86%, sem=1.0) than larvae without visual stimuli (avg=78%, sem=1.2, $p<0.0001$), Fig 6B. In addition, larvae exposed to the bouncing ball were more frequently oriented outward (avg=71%, sem=0.9), than larvae without visual stimuli (avg=67%, sem=0.8, $p<0.01$), Fig 6C. Thus, the response to the bouncing ball was very similar to the response observed in the one-fish assay. Larvae without visual stimuli were located in the same quadrant only 19.4% (sem=0.9) of the time, vs. 25% expected in a random distribution. Thus, larvae used in the two-fish assay showed no attraction to each other. Larvae exposed to the bouncing ball were together more frequently (avg=24%, sem=1.1, $p<0.01$), than the control larvae without visual stimuli (Fig 6D). To assure that this behavior was not caused by errors in the automated analysis when two larvae touch or overlap, we manually analyzed a 2-fish assay (12 wells, imaged for one hour, using a 60 sec interval). These manual analyses revealed that larvae were touching or overlapping only 0.8% of the time (sem=0.4%, n=12 wells). Based on these results, we

conclude that 7 dpf larvae have no social preference for being near each other. Our results are consistent with previously observed avoidance behaviors of larvae in the smaller wells of a 12 well plate [20], and with a recent study showing that robust shoaling behaviors in zebrafish develop later, during the juvenile stages [22]. The additional data points that were obtained by using two fish per well reduced the well-to-well variability and improved the statistics of the assay. Highly significant differences were observed in response to the bouncing ball stimulus. However, these differences were not significant when analyzing 12 wells per experimental group. We conclude that the two-fish assay can be used to measure avoidance of the bouncing ball and study avoidance between larvae, although the number of wells that would need to be imaged is a limiting factor for high-throughput studies.

3.5 The five-fish bouncing ball assay

We aimed to further reduce well-to-well variability by imaging groups of 5 zebrafish larvae in a single well (Fig. 7). The selection of 5 fish in the 5-fish assay is a tradeoff between a larger number of larvae, which would reduce well-to-well variability, and a smaller number of larvae, which would be beneficial for medium- and high-throughput applications. We found that, in the absence of visual stimuli, larvae were distributed randomly in the well, i.e. the larvae were down in the bottom half 50.9% of the time (sem=1.6, n=24 wells). When exposed to a blue bouncing ball, the larvae were down in the well 60.3% of the time (sem=1.6, n=36 wells). This difference is significant ($p<0.001$) when comparing all wells. A red bouncing ball was effective as well, i.e. larvae exposed to a red bouncing ball were 63.3% down in the well (sem=1.8, n=24 wells), which is significantly higher than the 50.9% (sem=1.6, n=24 wells) in the control group without visual stimuli ($p<0.0001$). In the latter case, the difference was significant when comparing 12 wells without visual stimuli vs. 12 wells with a red bouncing ball (the median p -value <0.05).

3.6 Bouncing, blinking, and stationary balls

To determine whether the larvae respond to the color or to the movement of the bouncing ball, we imaged the larval response to a stationary ball in the upper half of the well (Fig 7). The larvae exhibited a modest response to the stationary balls that was not significantly different from the no-ball controls. Larvae exposed to a stationary blue ball in the upper half of the well spent 54.9% of their time down in the well (sem=2.1, n=12 wells, $p>0.05$, vs. the no-ball control). Similarly, larvae did not display a significant response to a stationary red ball (avg=56.0% down, sem=2.2, n=12 wells, $p>0.05$, vs. no-ball control). When the larvae were exposed to a blue bouncing ball in the upper half of the well and a blue stationary ball in the lower half of the dish, larvae were down in the well 62.3% of the time (sem=2.7, n=12 wells, $p<0.01$, vs. no-ball control). Similarly, when the larvae were exposed to a red bouncing ball in the upper half of the well and a red stationary ball in the lower half of the well, larvae are down in the well 66.7% of the time (sem=3.0, n=12 wells, $p<0.001$ vs. the no-ball control). The experiments with a bouncing and stationary ball suggest that larvae respond to movement, rather than the color or size of the ball. In addition, these experiments show that it is possible to obtain an accurate measurement of avoidance behavior by imaging 12 wells with 5 larvae per well. The most effective stimulus is a red bouncing ball in the upper half of the well, counter-balanced by a red stationary ball in the lower half of the well (supplement 2).

The experiment with the red bouncing ball in the upper half of the dish and the stationary red ball in the bottom half of the dish was analyzed over time in 1 minute intervals. The response of the larvae to the bouncing ball did not diminish during the one-hour recording, indicating that the larvae did not habituate to the stimuli (Fig 7B). We repeated the experiment with the red bouncing ball and red stationary ball, adding an internal control. Larvae were first imaged for 30 minutes without visual stimuli and then for 30 minutes with

visual stimuli. Similar to the results described above, larvae exposed to a red bouncing ball and a red stationary ball show a significant avoidance response (avg=63.2% down, sem=2.1 vs. 49.5% down, sem=1.7 in the pre-stimulus control, n=24 wells, $p<0.0001$), Fig 7C. In contrast, larvae did not show a significant response to simultaneously blinking visual stimuli (avg=52.1% down, sem=1.8 vs. 51.3% down in the pre-stimulus control, n=24 wells, $p>0.05$) and did not show a significant response to the alternating blinking visual stimuli (avg=52.4% down, sem=2.6 vs. 50.7% down, sem 1.5 in the pre-stimulus control, n=24 wells, $p>0.05$), Fig 7C. For both the simultaneously and alternating blinking balls, the avoidance response of the larvae was significantly lower than the response to the bouncing ball ($p<0.001$ and $p<0.01$ respectively). These results show that the 7-day-old larvae specifically respond to movement, rather than local changes in light intensity.

3.7 Larval interactions in groups of five larvae

To examine more closely how larvae interact with each other in the five-fish bouncing ball assay, we imaged a 6-well plate at video speed (Fig. 8A). The videos revealed that larvae will occasionally congregate (Fig. 8B) and then quickly disperse (Fig. 8C). In addition, the larvae frequently swim in the same direction (Fig. 8D) and respond to the movement of other larvae in close proximity (Fig 8E). For example, in Figure 8E, larva 1 quickly darts away as the bouncing ball approaches. Larva 2 swims away from larva 1 and larva 3 moves away from larva 2, while larvae 4 and 5 rest at the edge of the well away from the activity. Based on the larval interactions in the two-fish and five-fish assays, we propose that the location of the larvae is influenced by two opposing forces: 1) the larvae avoid the bouncing ball, driving the larvae down towards the bottom edge of the well, and 2) the larvae prefer not to be near each other, causing a dispersion away from the bottom edge of the well (Fig 8F). The video recordings also show that there are numerous larval interactions and swimming patterns that remain to be analyzed in more detail and we plan to pursue these more complex analyses in future research.

4. Discussion

The present study describes a novel high-resolution imaging system for the automated analysis of behavior in zebrafish larvae. The developed system has several advantages over currently available imaging systems: (1) A single-camera unit can be built for approximately \$2,500 and is unique in its capacity to image 4 multiwell plates simultaneously. The cost of our system is 50–200 times less than the cost of an automated high-throughput microscope. In addition, automated microscopes are not designed for simultaneous imaging of multiple wells, i.e. the wells in a multiwell plate are imaged sequentially. This sequential imaging mode can be effective when imaging rapid behaviors in individual wells for short periods of time. For example, a 96-well plate can be imaged in an hour, when imaging the behavior of zebrafish embryos for 30–40 seconds per well [19]. However, automated microscopes are not suitable for high-throughput imaging of behavior over longer periods of time. A few companies, such as Noldus and ViewPoint, have developed effective macroscopic imaging systems for simultaneous imaging of all wells in a multiwell plate and these commercial imaging systems can be used to monitor activity levels in zebrafish larvae for several hours to several days on end (see for example [18]). Again, the imaging system and software described in this study compares favorably from a cost-per-plate perspective, i.e. our single-camera unit costs approximately 10 times less than a commercial macroscopic imaging system and has the capacity to image four multiwell plates instead of one. Thus, one advantage of the system described in this study is the low cost of imaging multiple multiwell plates. With a modest budget and a few days of time, it is possible to build a 3-camera system and image 12 multiwell plates simultaneously. (2) The system is also unique in its ability to project local visual stimuli inside the wells of a multiwell plate. A custom-built

system has recently been developed to provide local visual and electrical stimuli in 6 cm Petri dishes and this system can provide automated feedback to individual subjects in real time [23]. However, these state-of-the-art features are not available yet in commercial imaging systems that handle multiwell plates. (3) The visual stimuli can be removed from the images using the custom-written image analysis software. (4) The image analysis software can determine larval location as well as larval orientation, independent from swimming direction. The automated analysis of larval orientation has been described previously by individual laboratories [20, 21, 24, 25], but is not available yet in commercial systems. (5) The developed software can be used to automatically analyze multiple larvae per well, which is important when studying social behaviors. A disadvantage of our imaging system is that it is not designed to image at video speed for long periods of time. Commercial systems from ViewPoint and Noldus provide elegant solutions for imaging at video speed for several days, which can, for example, be important when studying circadian rhythms in activity [18]. Thus, the imaging systems are complementary in their capabilities. The commercial systems are well-designed to study larval activity, and the system described in this study is well-suited for imaging behaviors other than activity, including social interactions and the response to visual stimuli.

The developed imaging system was tested by measuring the response of 7-day-old zebrafish larvae to moving visual stimuli. Zebrafish larvae are known to swim away from large moving objects, which may function as a predator avoidance mechanism [12, 20, 21]. The present study shows that this behavior can be efficiently measured by projecting a 'bouncing ball' in each individual well of a 6-well plate. Zebrafish larvae will avoid the area where the bouncing ball is located. In addition, the larvae show increased frequencies of facing outward and swimming along the edge of the well. A preference for the periphery of an open field, or thigmotaxis, is a measure of anxiety in rodents [7, 26] and adult zebrafish [27–29], and it is likely that thigmotaxis and anxiety are linked in zebrafish larvae as well [12, 30]. Larvae also respond to the bouncing ball stimulus when it is counter-balanced with a stationary ball, indicating that the larvae respond to the movement of the visual stimulus, rather than its color or size. In contrast, larvae do not respond to blinking balls counter-balanced with a stationary ball, suggesting that the larvae are sensitive to movement, rather than local changes in light intensity. Finally, the different response to the bouncing and alternately blinking balls shows that the larvae are able to distinguish between these similar visual stimuli.

At present, the 5-fish assay is the most efficient approach for high-throughput screening, as it requires the smallest number of wells. It is also the most complex experimental paradigm, since the 7 dpf larvae avoid each other and avoid the visual stimuli. Screening strategies may be developed to combine the efficiency of the 5-fish assay with more detailed studies that separately examine the larval avoidance of large moving objects and avoidance of 'siblings'.

In conclusion, the high-throughput imaging system described in this study is unique in its capacity to simultaneously image multiple multiwell plates, and in its ability to provide local visual stimuli in the wells of multiwell plates. The system is easily duplicated and may be used to screen for genetic and environmental factors that cause developmental brain disorders. In addition, the system may be used to discover novel drugs to prevent or treat such disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Jain S, Heutink P. From single genes to gene networks: high-throughput-high-content screening for neurological disease. *Neuron*. 2010; 68:207–217. [PubMed: 20955929]
2. Carpenter AE. Extracting rich information from images. *Methods Mol Biol*. 2009; 486:193–211. [PubMed: 19347625]
3. Carpenter AE. Image-based chemical screening. *Nat Chem Biol*. 2007; 3:461–465. [PubMed: 17637778]
4. Bickel M. The beautiful cell: high-content screening in drug discovery. *Anal Bioanal Chem*. 2010; 398:219–226. [PubMed: 20577725]
5. Walter T, Shattuck DW, Baldock R, Bastin ME, Carpenter AE, Duce S, et al. Visualization of image data from cells to organisms. *Nat Methods*. 2010; 7:S26–S41. [PubMed: 20195255]
6. Zanella F, Lorens JB, Link W. High content screening: seeing is believing. *Trends Biotechnol*. 2010; 28:237–245. [PubMed: 20346526]
7. Crawley JN. Behavioral phenotyping strategies for mutant mice. *Neuron*. 2008; 57:809–818. [PubMed: 18367082]
8. Jhuang H, Garrote E, Yu X, Khilnani V, Poggio T, Steele AD, et al. Automated home-cage behavioural phenotyping of mice. *Nat Commun*. 2010; 1:68. [PubMed: 20842193]
9. Norton W, Bally-Cuif L. Adult zebrafish as a model organism for behavioural genetics. *BMC Neurosci*. 2010; 11:90. [PubMed: 20678210]
10. Gerlai R. High-throughput behavioral screens: the first step towards finding genes involved in vertebrate brain function using zebrafish. *Molecules*. 2010; 15:2609–2622. [PubMed: 20428068]
11. Cachat J, Stewart A, Grossman L, Gaikwad S, Kadri F, Chung KM, et al. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nat Protoc*. 2010; 5:1786–1799. [PubMed: 21030954]
12. Colwill RM, Creton R. Imaging escape and avoidance behavior in zebrafish larvae. *Reviews in the Neurosciences*. 2011; 22:63–73. [PubMed: 21572576]
13. Stewart A, Wu N, Cachat J, Hart P, Gaikwad S, Wong K, et al. Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. *Prog Neuropsychopharmacol Biol Psychiatry*. 2010
14. Gerlai R. Zebrafish antipredatory responses: a future for translational research? *Behav Brain Res*. 2010; 207:223–231. [PubMed: 19836422]
15. Cerutti, DT.; Levin, ED. Behavioral Neuroscience of Zebrafish Chapter 15. In: Buccafusco, JJ., editor. *Methods of Behavior Analysis in Neuroscience*. 2nd edition. Boca Raton (FL): CRC Press; 2009.
16. Ahmed O, Seguin D, Gerlai R. An automated predator avoidance task in zebrafish. *Behav Brain Res*. 2011; 216:166–171. [PubMed: 20674614]
17. Emran F, Rihel J, Dowling JE. A behavioral assay to measure responsiveness of zebrafish to changes in light intensities. *J Vis Exp*. 2008
18. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science*. 2010; 327:348–351. [PubMed: 20075256]
19. Kokel D, Bryan J, Laggner C, White R, Cheung CY, Mateus R, et al. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat Chem Biol*. 2010; 6:231–237. [PubMed: 20081854]
20. Creton R. Automated analysis of behavior in zebrafish larvae. *Behav Brain Res*. 2009; 203:127–136. [PubMed: 19409932]

21. Colwill, RM.; Creton, R. Automated imaging of avoidance behavior in larval zebrafish. In: Kalueff, AV.; Cachat, JM., editors. *Neuromethods, Vol 51 Zebrafish Neurobehavioral Protocols* Humana Press, Springer Protocols: 1st Edition. 2010. ISBN 978-1-60761-952-9
22. Buske C, Gerlai R. Shoaling develops with age in Zebrafish (*Danio rerio*). *Prog Neuropsychopharmacol Biol Psychiatry*. 2010
23. Blackiston D, Shomrat T, Nicolas CL, Granata C, Levin M. A second-generation device for automated training and quantitative behavior analyses of molecularly-tractable model organisms. *PLoS One*. 2010; 5:e14370. [PubMed: 21179424]
24. Burgess HA, Granato M. Modulation of locomotor activity in larval zebrafish during light adaptation. *J Exp Biol*. 2007; 210:2526–2539. [PubMed: 17601957]
25. McLean DL, Masino MA, Koh IY, Lindquist WB, Fetcho JR. Continuous shifts in the active set of spinal interneurons during changes in locomotor speed. *Nat Neurosci*. 2008; 11:1419–1429. [PubMed: 18997790]
26. Komada M, Takao K, Miyakawa T. Elevated plus maze for mice. *J Vis Exp*. 2008
27. Dadda M, Domenichini A, Piffer L, Argenton F, Bisazza A. Early differences in epithalamic left-right asymmetry influence lateralization and personality of adult zebrafish. *Behav Brain Res*. 2010; 206:208–215. [PubMed: 19765616]
28. Peitsaro N, Kaslin J, Anichtchik OV, Panula P. Modulation of the histaminergic system and behaviour by alpha-fluoromethylhistidine in zebrafish. *J Neurochem*. 2003; 86:432–441. [PubMed: 12871584]
29. Champagne DL, Hoefnagels CC, de Kloet RE, Richardson MK. Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): relevance for stress research. *Behav Brain Res*. 2010; 214:332–342. [PubMed: 20540966]
30. Colwill RM, Creton R. Locomotor behaviors in zebrafish (*Danio rerio*) larvae. *Behav Processes*. 2011; 86:222–229. [PubMed: 21147203]

Figure 1. Automated analysis of avoidance behavior in multiwell plates

A) We developed a novel high-resolution imaging system, using a tall cabinet, a 15 megapixel Canon camera, and a laptop with an LCD screen that holds four multiwell plates. B) The camera is mounted on the top shelf. C) The laptop's LCD screen is used to show PowerPoint presentations to the larvae. D) Four 6-well plates with 7 dpf zebrafish larvae are placed on the LCD screen. The larvae are exposed to blue and red balls that are bouncing from the left to the right and back in the upper half of the well. E) Full color image of a single well with blue bouncing ball. The images are opened in ImageJ, where the full color image is split into a red (F), green (G), and blue (H) channel. Note that the blue ball is not visible in the blue channel. I) Agarose rings and spherical aberrations can be filtered out by background subtraction. J) Larvae are separated from the background with a threshold for dark objects. The arrowhead indicates a larva and the arrow points to a small air bubble in the well. K) The larvae are separated from smaller objects using ImageJ's particle analysis.

L) Diagram of a larva, illustrating the centroid, center of mass, and center of the bounding rectangle. Scale bars = 1 cm.

Figure 2. ImageJ macro for the automated analysis of larval behavior

The ImageJ macro includes dialog boxes for the number of images, number of wells, color, background subtraction, particle size, and threshold. The regions of interest and thresholds are set in the first image and are then applied to all subsequent images. The software automatically analyzes up to 12 wells in a series of images and multiple larvae can be identified in a single well. The measurements of individual larvae are saved in a results file, which includes the image name, larval area in pixels, mean intensity, X and Y coordinates of the centroid, X and Y coordinates of the center of mass, X, Y, width, and height coordinates of the bounding box, well number, and the X, Y coordinates of the midpoint of the well. The results file can be opened in MS Excel for further analysis. The ImageJ macro is available in the supplementary information (supplement 3).

Figure 3. Video analysis of larval swimming patterns

A–D) Swimming patterns of 7 dpf larvae without visual stimuli. Larvae utilize the top and bottom halves of the well, swim along the edges and center, and rest, without a clear preference for a specific location in the well. E–H) Swimming patterns of 7 dpf larvae exposed to a bouncing ball. Larvae turn away from the bouncing ball, either rapidly (E) or slowly (F), swim erratically until reaching the edge of the well (G), or rest in the upper or lower halves of the well (H). Each panel is a 30 second video recording of a single larva, analyzed in ImageJ at 5 frames per second. To display the swimming patterns, the 150 frames were collapsed into a single image.

Figure 4. Optimization of the image interval

7 dpf larvae exposed to a blue bouncing ball were imaged for one hour using a 6 second interval between images (1 fish per well, 11 wells total). The data set was then analyzed at a 6, 12, 18, 24, 30, and 60 second interval. A) The measured swim speed decreased when using longer intervals between images. B) The percentage of larvae that are down in the dish did not show a significant change when imaging at longer intervals. C) Similarly, the percentage of larvae located at the edge of the well did not show a significant change when imaging at longer intervals. The error bars represent the standard error of the mean (s.e.m.).

Figure 5. Quantification of behavior in the one-fish bouncing ball assay

7 dpf larvae, one per well, were imaged without visual stimuli ('Control') or with a blue bouncing ball in the upper half of the well ('BB'). A) Larvae exposed to the bouncing ball spent significantly more time down in the dish, away from the bouncing ball, than larvae without visual stimuli. B) Larvae exposed to the bouncing ball spent significantly more time at the edge of the dish. C) Scatter plot showing the location of larvae in 12 wells during a one hour exposure to the bouncing ball. Note that the majority of the larvae are located along the edge, down in the well. D) Larvae exposed to the bouncing ball spent significantly more time facing outward towards the edge of the well. E) We did not observe a statistical difference in speed between the control larvae and the larvae exposed to the bouncing ball. However, larvae exposed to the bouncing ball move significantly faster when they are in the upper half of the well (BBU) vs. the lower half of the well (BBD). F) We did not observe a statistical difference between the percent rest in the control larvae and the larvae exposed to the bouncing ball. However, larvae exposed to a bouncing ball rest more frequently down in the dish (BBD) than they do up in the dish (BBU). Larvae are considered to be at 'rest' when they move less than 5 mm in a one minute interval. The larvae were imaged for one hour using a 60 second interval between images. u=up, d=down, e=edge, c=center.

Figure 6. Quantification of behavior in the two-fish bouncing ball assay

7 dpf larvae, two per well, were imaged without visual stimuli (Control) or in the presence of a blue bouncing ball (BB). A) Larvae exposed to the bouncing ball spent more time down in the dish than larvae without visual stimuli. B) Larvae exposed to the bouncing ball spent more time at the edge of the well than larvae without visual stimuli. C) Larvae exposed to the bouncing ball were more frequently oriented outward than larvae without visual stimuli. D) Larvae without visual stimuli were located in the same quadrant less frequently than expected in a random distribution. Larvae exposed to the bouncing ball were together more frequently, than the control larvae without visual stimuli. The larvae were imaged for one hour using a 60 second interval between images.

Figure 7. Avoidance behavior in the five-fish bouncing ball assay

7 dpf larvae, five per well, were imaged without visual stimuli (Control) or in the presence of various stimuli, including a bouncing ball (BB) and/or a stationary ball. **A)** Control: larvae without visual stimuli were distributed evenly in the upper and lower halves of the well. **Blue BB:** larvae exposed to a blue bouncing ball in the upper half of the well spent significantly more time in the bottom half of the well. **Red BB:** larvae exposed to a red bouncing ball in the upper half of the well, spent significantly more time in the bottom half of the well. **Blue Ball:** larvae did not display a significant response to a stationary blue ball. **Red Ball:** larvae did not display a significant response to a stationary red ball. **Blue BB and Blue Ball:** larvae accumulated down in the dish when exposed to a blue bouncing ball in the

upper half of the dish and a blue stationary ball in the bottom half of the dish. Red BB and Red Ball: larvae accumulated down in the dish when exposed to a red bouncing ball in the upper half of the dish and a red stationary ball in the bottom half of the dish. The larvae were imaged for one hour using a 60 second interval between images. B) The experiment with the red bouncing ball in the upper half of the dish and the stationary red ball in the bottom half of the dish was analyzed over time in 1 minute intervals (n=12 wells). The dotted line indicates the expected 50% down in a random distribution. C) Larvae were first imaged for 30 minutes without visual stimuli and then for 30 minutes with visual stimuli. Similar to the results shown above, larvae exposed to a red bouncing ball (BB) and a red stationary ball showed a significant response. In contrast, simultaneous blinking balls (SIM) or alternating blinking balls (ALT) did not show a significant response. The larvae were imaged for one hour total, using a 30 second interval between images.

Figure 8. Video analysis of larval interactions

7 dpf larvae, 5 per well, were imaged for 15 minutes at 30 fps in the presence of a bouncing and stationary ball. A) Color image of the five-fish assay with a bouncing ball in the upper half and a stationary ball in the lower half of the dish. B) Snapshot of larvae accumulating in the bottom half of the well. C) The larvae quickly disperse in a 6 second period following the accumulation shown in panel B. D) four larvae move together along the same path over a 6 second interval. E) Larva 1 quickly moves away from the bouncing ball, setting off a cascade of movements in a 6 second period. Larva 2 moves when larva 1 approaches, and larva 3 moves when larva 2 approaches. F) Model of the swimming patterns of zebrafish larvae. Zebrafish larvae avoid large moving objects and swim towards the bottom of the dish (orange arrow). Larvae that are down in the dish move apart and disperse away from each other (blue arrows).