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Zebrafish Neurobehavioral Protocols

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Preface to the Series

Under the guidance of its founders Alan Boulton and Glen Baker, the Neuromethods series by Humana Press has been very successful since the first volume appeared in 1985. In about 17 years, 37 volumes have been published. In 2006, Springer Science + Business Media made a renewed commitment to this series. The new program will focus on methods that are either unique to the nervous system and excitable cells or which need special consideration to be applied to the neurosciences. The program will strike a balance between recent and exciting developments like those concerning new animal models of disease, imaging, in vivo methods, and more established techniques. These include immunocytochemistry and electrophysiological technologies. New trainees in neurosciences still need a sound footing in these older methods in order to apply a critical approach to their results. The careful application of methods is probably the most important step in the process of scientific inquiry. In the past, new methodologies led the way in developing new disciplines in the biological and medical sciences. For example, Physiology emerged out of Anatomy in the nineteenth century by harnessing new methods based on the newly discovered phenomenon of electricity. Nowadays, the relationships between disciplines and methods are more complex. Methods are now widely shared between disciplines and research areas. New developments in electronic publishing also make it possible for scientists to download chapters or protocols selectively within a very short time of encountering them. This new approach has been taken into account in the design of individual volumes and chapters in this series.

Wolfgang Walz

Preface

The use of zebrafish (*Danio rerio*) in neurobehavioral research has dramatically increased over the past decades. This has led to the development of novel behavioral assays to quantify a variety of behaviors seen in larval and adult zebrafish. There has also been an increasing trend toward the use of automated video-tracking software to analyze the behaviors observed in these assays. The ability to correlate behavioral patterns with physiological endpoints on an individual is another advantage of using zebrafish in neurobehavioral research. As such, zebrafish are rapidly emerging as a promising, high-throughput animal model for biomedical research.

The present book is written by the leading experts in zebrafish research, many of which are active members of the Zebrafish Neuroscience Research Consortium (ZNRC). This volume is composed of protocols detailing three major research areas, including (1) the use and interpretation of video-aided quantification of zebrafish behaviors, (2) descriptions of novel assays commonly used to quantify emotionality, as well as learning, memory, and social behaviors in zebrafish, and (3) the quantification of circulating cortisol levels and the subsequent correlation to anxiety-like behaviors in zebrafish. This book will serve as a useful *practical* complement to another book of this series, *Zebrafish Models in Neurobehavioral Research*, which covers scientific/theoretical problems and neurobiological principles of zebrafish behavior.

The first chapter of the present book describes the principles of video-tracking in zebrafish research, making note of the advantages of video analysis. These include the ability to obtain an increased number of behavioral endpoints, many of which are not quantifiable using traditional observation techniques, as well as improved precision when quantifying certain zebrafish behaviors that are otherwise highly subjective. In line with this, Chapter 2 presents a novel approach to analyze data produced with automated behavioral recording. Termed the "videogram", this single image forms a density map of zebrafish activity in a video sequence, serving as a direct, repeatable, and unbiased measure of animal activity.

Chapter 3 explains how automated video-tracking technologies can be connected with a behavioral assay in larval zebrafish. Focusing on the quantification of avoidance behaviors in larva, this protocol describes two assays, the "bouncing-ball assay" and the "two-fish assay", which measure responses to a threatening stimulus as well as avoidance behavior, respectively.

The following chapters of this book describe more specific behavioral paradigms to examine the wider behavioral repertoire of zebrafish. This includes Chapter 4's quantification of zebrafish responses to chemical alarm cues or substances that indicate the presence of predation risk. Several assays of zebrafish learning and memory are summarized in the subsequent chapters, including a modified T-maze test of the acquisition and extinction of reward-visual stimulus association, simple conditioned place preference assays for assessing the reinforcing properties of drugs of abuse, as well as a method for studying conditioning olfactory behaviors in adult zebrafish. Additionally, Chapter 8 provides a detailed protocol for a light/dark plus maze novel environment test, which measures thigmotaxis and scototaxis in order to assess anxiety-like behaviors in zebrafish.

Chapters. 9 and 10 are logically interconnected and describe assays of zebrafish social behavior. The first contribution describes methods for simple, fast, and accurate

assessment of drug-induced effects on social and motor behaviors in zebrafish. Such behavioral paradigms that may be particularly useful in conjunction with high-throughput drug screening. The second protocol outlines an assay for identification, characterization, and quantification of agonistic behaviors in zebrafish, which can be used to quantify the effects of pharmacological and genetic manipulations in this species.

Chapter 11 provides a protocol for determining circulating cortisol levels in zebrafish. Such physiological quantification is highly applicable to behavioral measures of fear and anxiety-like responses in zebrafish, as the zebrafish acute stress response is analogous to that of humans, resulting in increased production and secretion of cortisol into the blood.

Chapters. 12 and 13 provide some further protocols that can be used to phenotype zebrafish behavior in novelty tests. The first protocol explains how to analyze an interesting behavioral pattern recently observed in zebrafish – their natural tendency to form preferred safe zones, or "homebases". The second assay parallels Chapter 8 and is based on the fish's inherent tendency of scototaxis. This chapter illustrates, using two variations of the light/dark box test, how this simple paradigm can be used to assess zebrafish anxiety-like behavior evoked by anxiogenic or anxiolytic drug administration.

While most drugs are administered to zebrafish by immersion of a fish into a drug solution, Chapter 14 discusses an alternative method of drug administration, which utilizes intraperitoneal injection to treat zebrafish with a pharmacological agent. This protocol is especially useful for those working with drugs that may not be conducive for immersion, such as insoluble or highly toxic compounds.

The previous chapters are further complemented by Chapter 15, which instructs the reader on how to employ Maximum Predictive Value (MPV) to determine how sensitive a particular model is to various pharmacological manipulations. As a particular example, this chapter outlines how to utilize this measure to validate behavioral endpoints in the novel tank test when assessing anxiety-like behavior.

The final Chapter 16 presents a highly innovative approach to zebrafish behavior based on three-dimensional reconstructions of zebrafish swim traces to better understand their behavior. This protocol logically summarizes other chapters in this volume, providing a methodology for using video-tracking technology to more comprehensively characterize zebrafish behavior. This contribution will be especially useful for analyzing automated endpoints for drug- and class-specific zebrafish phenotypes in parallel.

Overall, as the reader will learn from this book, zebrafish offer an excellent opportunity to perform steadfast scientific investigations in a robust and high-throughput manner. All this remarkably separates the zebrafish from other classical laboratory animals. Moreover, the relative ease at which zebrafish can be housed, reproduced, and handled has prompted their introduction into teaching laboratories. Given the value of zebrafish in the research and teaching laboratories, we hope that this book will be accessible to a wide range of expertise. The Editors acknowledge the important role of ZNRC in promoting zebrafish research, including many protocols described here by active participating laboratories. The present compilation of neurobehavioral protocols is particularly timely, as it provides the first practical introduction to the exciting field of zebrafish behavioral research. Perhaps even more importantly, all assays described herein can be performed, creatively modified, further improved, and combined in almost limitless ways, again epitomizing the growing potential of zebrafish in modern scientific inquiry.

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Chapter 1

Video-Aided Analysis of Zebrafish Locomotion and Anxiety-Related Behavioral Responses

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Abstract

Due to physiological and anatomical similarities to other vertebrates, zebrafish are becoming a widely used model in neurobehavioral research. With the growing popularity of zebrafish as experimental subjects, it is important to develop tools that accurately record their behavioral phenotypes. Video-aided analysis of zebrafish behavior offers an increased spectrum of behavioral endpoints, some of which are not available using traditional (manual) observation. In addition, the use of computer software allows quantification of certain zebrafish behaviors that are otherwise highly subjective. This protocol describes a method for fast, accurate, and consistent video-aided measurements of zebrafish locomotion and anxiety-related behavior.

Key words: Behavioral endpoint, manual observation, video recording, video-tracking software, human error, novel tank.

1. Introduction

Zebrafish have long been utilized as an experimental animal model for biomedical research, particularly in developmental and genetic studies (13) and drug discovery (8). Several characteristics contribute to the utility of zebrafish models. Being a constantly active animal that readily acclimates to new environments, zebrafish make an excellent species choice for behavioral experiments (12). Additionally, zebrafish have a low maintenance cost,

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a robust reproductive cycle, rapid development, and a large number of offspring (7).

Zebrafish are also becoming commonly used in neuroscience research (10). Until recently, the recording of zebrafish behavior was performed manually, making it vulnerable to human error. Human errors and inter/intra-rater variability can lead to incorrect data acquisition and interpretation, thereby reducing the validity of an experiment. Computerized video-tracking tools that record zebrafish movements provide standardized observation of behavioral endpoints and reduce human errors. Eliminating the discrepancies caused by manual observation of zebrafish behavior allows for a more regulated standard of data recording and promotes experimental soundness and reproducibility. Another benefit of using the video-tracking program is that instead of relying on a single chance to manually observe every behavioral endpoint, videos can be stored, replayed, and reanalyzed.

The setup of the video-tracking system is essential for recording the zebrafish movements. For example, variations in lighting may hinder the program's ability to detect and analyze the fish. It is, therefore, important to determine an appropriate background for video-tracking with adequate light and that these conditions are standardized for all subjects. It should be noted that the video-tracking system is less reliable in analyzing erratic movements (**Table 1.1**). A methodological problem can also arise if the video-tracking system fails to record the fish for an unknown reason (such as multiple shadows or spastic water movement, which interfere with the programs' ability to recognize the fish), as this may skew the results. The present protocol outlines the video-tracking approach to analysis of zebrafish behavioral phenotypes. A more detailed description of zebrafish video-tracking analyses is provided in our recent publication (5).

2. Materials

Adult zebrafish (purchased from a commercial distributor) must be experimentally naïve, and given at least 10 days to acclimate to the laboratory environment (e.g., the room and water temperature maintained at 25–27°C with illumination provided by ceiling-mounted fluorescent light tubes). The video-tracking programs used here to record zebrafish movements are TopScan (TopView Animal Behavior Analyzing System) from CleverSys Inc. (Reston, VA) and Ethovision® XT7 from Noldus Information Technology (Netherlands). However, other video-tracking programs may also be used in zebrafish neurobehavioral research.

Table 1.1
Summary of behavioral endpoints and their signficance measured in the novel tank diving test (a – automatic observation, m – manual observation, c – calculations based on manually or automatically recorded data)

Endpoint (units)	Registration	Definition	Interpretation
Latency to enter the top (s)	m,a	The amount of time to first cross (by the center of mass of the body) from the defined bottom portion to the top of the novel tank	When introduced to a novel environment, zebrafish naturally dive to the bottom of the tank and gradually explores as it habituates to the test apparatus. The longer latency indicates higher anxiety levels
Time spent in top (s)	m,a	Total time spent in the top portion of the novel tank	A longer duration in the top of the tank indicates lower anxiety levels
Time spent top:bottom ratio	c	The ratio of the time spent on top over bottom	Lower ratio indicates higher anxiety level
Number of entries to the top	m,a	The number of crosses from the defined bottom portion to the top of the novel tank	More top entries indicate lower anxiety levels
Entries top:bottom ratio	c	The ratio of the number of entries to the top over bottom	Lower ratio indicates higher anxiety level
Average entry duration (s)	c	The amount of time spent at the top of the novel tank during each crossing	Calculated as time spent in the top divided by the number of entries to the top. Shorter average entry duration indicates higher anxiety level
Distance travelled in the top (m)	a	Total distance traveled in the defined top portion	Zebrafish with high anxiety would travel more distance in the bottom of the tank
Distance travelled top:bottom (m)	c	A ratio of the total distance traveled in the defined top portion versus the defined bottom	A lower top:bottom ratio indicates a higher stressed fish

Table 1.1 (continued)

Endpoint (units)	Registration	Definition	Interpretation
Total distance traveled (m)	a	Total distance the zebrafish traveled within the novel tank	Reflects general motor/neurological phenotypes. Zebrafish are generally quite sensitive to non-specific motor impairments and sedative drug effects (*see* **Section 11**)
The number of erratic movements	m,a	Sharp or sudden changes in direction of movement or repeated darting behavior	Indicates increased fear/anxiety, and are generally higher in stressed zebrafish
Average velocity (m/s)	a	Magnitude and direction of zebrafish speed	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Freezing bouts (frequency)	m,a	Total immobility(>1 s), except for the eyes and gills	Indicate increased anxiety and are generally higher in stressed zebrafish
Freezing duration (s)	m,a	Total duration of all freezing bouts	Indicates increased anxiety and is generally higher in stressed zebrafish
Meandering (°/m)	a	The degree of turning (vs. straight locomotion)	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Turning angle (°)	a	Total turning angle	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Angular velocity (°/s)	a	Magnitude and direction of zebrafish angular speed	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test

3. Experimental Setup

After pre-treatment, zebrafish are placed individually in a 1.5-L trapezoidal tank (e.g., 15.2 height×27.9 top×22.5 bottom×7.1 width cm; Aquatic Habitats, Apopka, FL) maximally filled with aquarium treated water. Novel tanks rest on a level, stable surface and are divided into two equal virtual horizontal portions, marked by a dividing line on the outside walls. Once zebrafish are relocated to novel tanks, swimming behavior is recorded by two trained observers (inter-rater reliability >0.85) and by the video-tracking system over a 6-min period (**Fig. 1.1**) (5).

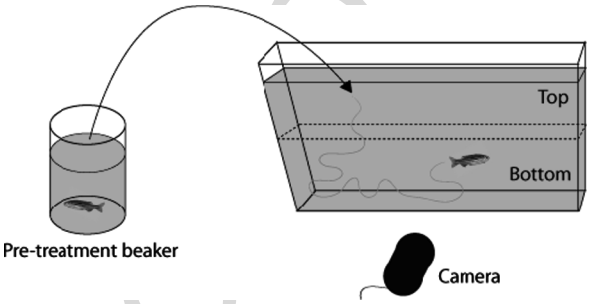

Fig. 1.1. Novel tank diving test. Zebrafish are exposed to the experimental challenge in a pre-treatment beaker before being transferred (via net) into the novel tank for behavioral observation and phenotyping. Control groups undergo same procedures without challenge in pre-treatment beaker.

4. Behavioral Endpoints

During the novel tank diving test, video-tracking programs can analyze the number of times the zebrafish entered the top of the novel tank, duration in the top/bottom, duration proportion in top/bottom, distance (m) traveled in top/bottom, latency to enter the top (s), velocity (m/s), total distance traveled, and information about erratic movements and freezing bouts (frequency, duration). Detailed definitions of each endpoint can be found in **Table 1.1**. Once all the data have been collected and analyzed, comparison of the control and experimental groups can be performed (if necessary, they may also be paralleled with physiological (e.g., endocrine) analyses; *see* **Chapter 11** on cortisol assay in this book).

5. Time Requirement

The time required for the protocol varies depending on the number of animals per group and the number of experimental groups, and is based on zebrafish locomotor activity levels. In general, zebrafish behavior assessment will last 6 min per animal. Depending on the amount of behavioral data collected, analysis may take between 2 and 4 days.

6. Data Analysis

To analyze the data, researchers may use the Mann-Whitney U-test for comparing two groups (parametric Student's *t*-test may be used if data are more normally distributed) or an analysis of variance (ANOVA) for multiple groups, followed by a post hoc test. More complex designs, such as a one-way ANOVA with repeated measures (time) or *n*-way ANOVA (additional factors: time, treatment, genotype, stress, sex, etc.), can also be used in zebrafish behavioral studies.

7. General Procedure

Zebrafish are transported individually from their home tank to the novel tank with careful handling to reduce net-stress. Recording starts and continues for a period of 6 min. Following the testing period, the animals are removed from the novel tank and can be reintroduced to their original tank for further experimentation or dissected for tissue harvesting and collection of biological data. Each zebrafish is given a subject number and the recorded video file name is changed accordingly, to correlate to that zebrafish number (Note: The recorded video file must be in MPEG format for video-tracking software to analyze it). The recorded videos are then imported into TopScan or EthoVision to be analyzed.

8. TopScan

TopScan is able to provide several important endpoints unavailable to human observation, such as total distance traveled, distance traveled in the top/bottom portion, velocity, and a traceable path of the subject's swimming pattern.

1. The first protocol step required for TopScan video analysis is to open the selected video for analysis.
2. Next, a background frame must be set, a required prerequisite for video analysis that can be achieved by finding the "Background" tab and clicking on "Set this frame as background".
3. The protocol then requires the setting of the arenas/parameters. Go to "Design" to set the top and bottom arenas by using the "polygon" tool under the "Arena Design Tools."
4. After setting the arenas, they must be activated. The investigator goes to the "Area" section of the "Event" and clicks on the top and bottom arenas (one at a time) to activate it.
5. The final step in the protocol requires the "Analyze" box to be checked for the analysis. The "Analyze" function calculates every movement that the zebrafish made.
6. After the analysis is performed, all data are exported to Microsoft Excel, to be compared and statistically evaluated. More detailed information about TopScan can be obtained from http://www.cleversysinc.com.

9. EthoVision® XT7

EthoVision® XT7 provides tracking and analysis of parameters such as path and distance traveled, velocity, meandering, and angular velocity (**Table 1.1**). This is an established user-friendly program that facilitates observation and analysis of behavioral endpoints while minimizing human error. The protocol for this program is as follows.

1. Open EthoVision and click "New Experiment." The experiment should be named with an appropriate description.
2. Desired detection features and units should be selected. Video files are moved into the "Media Files" sub-folder in the newly created experimental folder. A "Trial List" is created with the following variables: fish group, group ID, and fish ID. Next, the arena settings are adjusted.
3. To set the background, capture the image prior to introduction of the fish into the novel tank. Then use a square or rectangle tool to define the entire novel tank as Arena 1. Divide this Arena at the midline into defined Top and Bottom Zones.
4. Calibration and validation of arena settings are then performed. Detection settings should be adjusted ensuring that the subject is darker than the background image.

5. Save the settings and check the box for "Track Smoothing". Next, press "Play".
6. Videos are then analyzed. When analyses are complete, enter independent variables (Group, GroupID, FishID) for trial and collected data.
7. To export data into Excel for statistical analysis and further comparison, go to the "Export" menu and select "Analysis Data." The Settings screen will appear. Type the name of the appropriate destination folder in the field for "File name prefix". Under "File Type," select Excel and click "Start Export". More information on EthoVision® XT can be obtained from www.noldus.com.

10. Anticipated/ Typical Results

10.1. Observation Comparability

We anticipate the comparisons of data produced by the video-tracking system with those produced by manual observation to show a significant correlation between the two approaches. Indeed, our own recent data (**Fig. 1.2**) demonstrate high (>80–90%) correlation between the two methods (6) for most of the major parameters assessed, confirming that the video-tracking system is a reliable tool for zebrafish neurobehavioral research.

10.2. The Novel Tank Diving Test

The novel tank diving test exploits the stress response and allows comparison of anxiety-induced behavior in experimental versus control groups. **Figure 1.1** illustrates how this test is employed in an experimental design. When the zebrafish is exposed to a novel environment, it initially dives to the bottom and then gradually explores the top. Inhibited exploratory movement, reduced speed, and increased frequency of escape-like erratic behaviors are associated with higher levels of anxiety elicited by different stressors (3, 9, 11) (**Table 1.1**). These behaviors are highly sensitive to pharmacological treatment, as zebrafish exploration is increased after treatment with anxiolytic drugs, including benzodiazepines, SSRIs, nicotine, and ethanol (1, 3, 4, 9). Conversely, stressful stimuli (e.g., predator exposure or alarm pheromone, anxiogenic drugs, and drug withdrawal) have been shown to increase anxiety-like behavior in this paradigm, leading to longer latency to explore the upper half of the novel tank, less time in the top, more erratic movements, and longer/more frequent freeze bouts (2, 3, 11). **Figure 1.3** illustrates typical results observed in the novel tank diving test after exposure to anxiogenic acute caffeine. This simple yet high-throughput test can be used as an approach in quickly and accurately identifying the biomarkers linked to a disorder and in screening the efficacy of different pharmacological treatments.

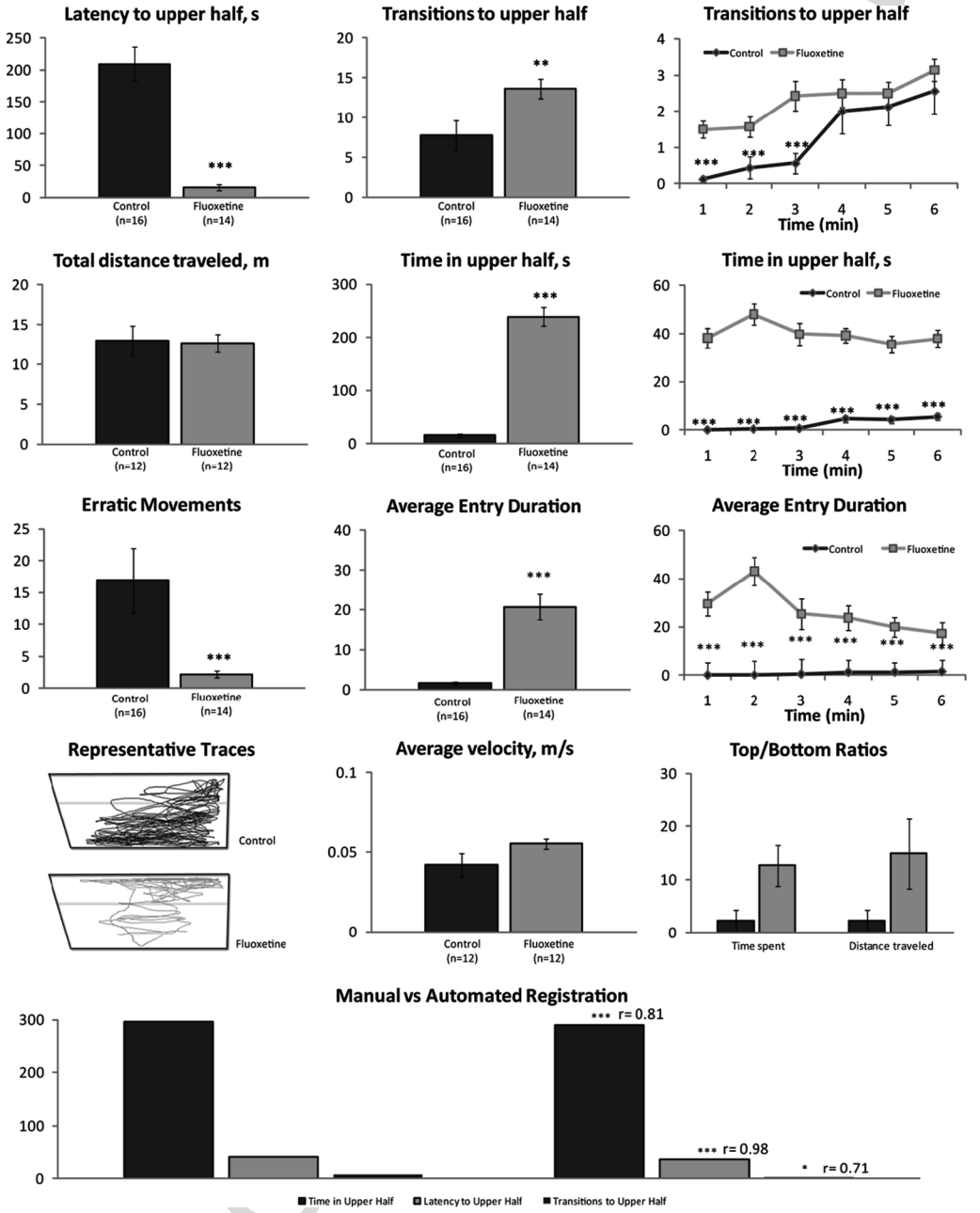

Fig. 1.2. Anxiolytic effects of chronic fluoxetine (100 μg/L, 2 weeks in the home tanks) on zebrafish behavior in the novel tank diving test, based on manual behavioral phenotyping and automated behavioral characterization with video-tracking software (CleverSys Inc). Data are presented as mean ± SEM, *p<0.05, ***p<0.005 versus control, U-test (5, 6).

10.3. Strain Differences

Using the video-tracking approach, we found robust observable strain differences in the novel tank diving test. As can be seen in **Fig. 1.4**, the wild-type zebrafish exhibited greater exploratory behavior (compared to the leopard mutant strain), suggesting

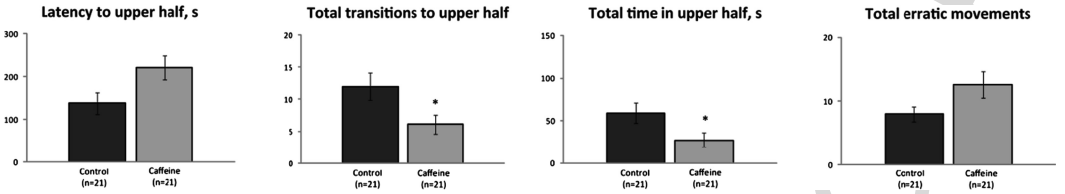

Fig. 1.3. Anxiogenic effects of acute caffeine (100 mg/L, 15 min pre-exposure time) on zebrafish behavior in the novel tank diving test, based on automated behavioral characterization with video-tracking software (CleverSys Inc). Data are presented as mean ± SEM, $^{*}p<0.05$ versus control, U-test (6).

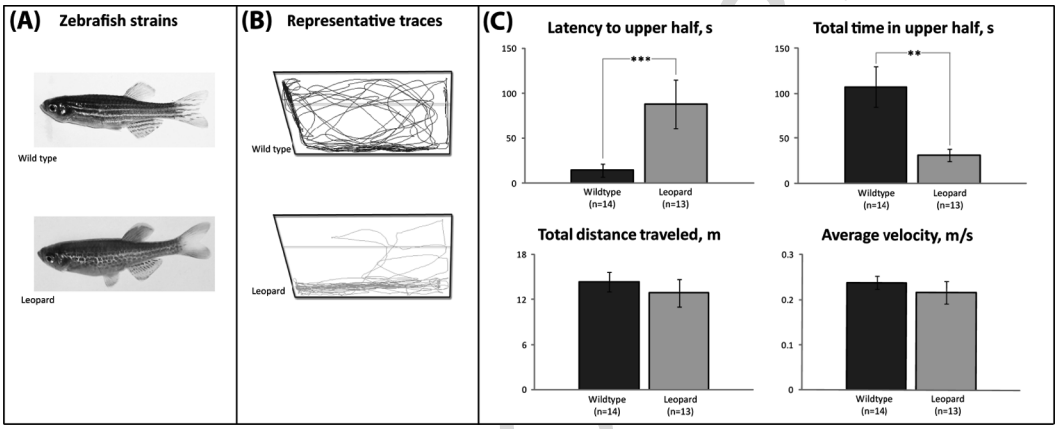

Fig. 1.4. Strain differences in zebrafish novel tank diving test behavior. Two different strains of zebrafish used in this study (**a**) display specific patters of their exploratory behavior, as illustrated by representative swimming traces (**b**) and measured behavioral endpoints (**c**), which were analyzed using video-tracking software (CleverSys Inc), $^{**}p<0.01$, $^{***}p<0.005$, U-test (6).

higher baseline anxiety levels in the mutants than the wild type. Due to the behavioral differences amongst zebrafish strains, strain selection must be taken into consideration when choosing and comparing zebrafish for experimentation.

11. Troubleshooting (The Following Generally Applies to All Video-Tracking Software Programs)

11.1. Software Not Detecting Fish

The problem with detection of the object (fish) by software can be resolved by altering one or several settings: detection settings, lighting, and background. For example, if the software detects the glare or another object as the experimental object, changing

the contrast between the object and the background in Detection Settings can offer a solution. If the problem still persists, consider using another detection method available (e.g., Differencing, Dynamic subtraction (Ethovisions XT7 has a re-do option), Gray scaling, or Static subtraction). If different detection methods have been employed, but the software still cannot detect the fish, this may be a video-related problem. Adequate lighting is necessary. If the video is too dim or too bright, the lighting of the setup must be adjusted prior to recording. Too dim or too bright lighting will make it harder for the system to differentiate the subject from the background, and hence the subject may remain undetectable during the analysis. Using a solid color as a background will also help decrease the chance of misdetection of the subject.

11.2. Software Losing Fish in the Middle of the Video

The software may detect something else as the fish in the middle of the video. For example, this is usually caused by a glare in the video. A simple adjustment in detection setting, such as contrast, will likely resolve this problem.

11.3. Behavioral Endpoints Collected Do Not Reflect Actual Behavior

Make sure that the arenas and the zones were properly defined, calibration is accurate, and the endpoint's "rules" were identified clearly. For example, for zone transition, make sure the endpoint is set from "bottom" to "top" instead of "top" to "bottom", if the endpoint is measuring how many times the subject enters the top.

11.4. An Error Occurred During the Acquisition of a Trial

During acquisition, unexpected errors will occur. For example, the software may lose detection of the fish, or the experimenter may use a wrong video for the trial. Fortunately, reanalysis of the video is possible. However, if the video was recorded with another program, a new trial must be added. Simply delete the error/unwanted trial, and add a new one to start over (Ethovisions XT7 has a re-do option).

11.5. The Video Program Is Running Very Slow

Typically, a hardware problem. Make sure that the computer meets the minimum program requirements. Also, turn off tracing option during playback/analysis of data.

11.6. Unable to Define Zones

Zone definition (i.e., top half vs. bottom half, or center zone vs. border zone) may be undefined or defined incorrectly according to the video-tracking program. The solution for this problem is to investigate the arena setting and calibrations (found under the "Setup" tab) to see if they are correct or need to be readjusted.

11.7. Video-Tracking Program Unable to Play Video

Video-tracking programs analyze videos under specific formats. The file must be converted into the specific required (e.g., MPEG, AVI) file type.

11.8. Calculation of "Distance to Zone" in the Analysis Yields Either 0.00, or No Results at All

The calculation of "distance to zone" is found under the analysis tab. Calculations that yield 0.00 are specifically due to improper calibration of the zones. To fix this problem, simply recalibrate the zones and also check that the arena itself is not selected, instead of a specified zone.

11.9. Role of Memory and Conditioned Responses

Zebrafish demonstrate good learning and memory phenotypes. For example, conditioned responses can develop after a single trial and a response can be obtained even when there is a time delay of several minutes between the presentation of the unconditioned and conditioned stimuli (12). Conditioned responses can also be passed on to naïve fish in a process known as social facilitation. The naïve fish will display a conditioned response in the presence of a previously conditioned fish, and will retain this learned response when solitary or in the company of a new group of naïve fish (12). Likewise, fish can recall training for up to a 10 day period (12). Collectively, this implies a good memory capability for this animal. Therefore, re-testing zebrafish in the novel tank should be avoided. If this is not possible, a longer interval (e.g., >3 weeks) between trials must be allowed in order to minimize potentially confounding data.

11.10. Zebrafish Display Abnormally High or Low Levels of Locomotion

It may be a strain-specific phenomenon, and the researchers may need to re-assess the strain's suitability for the experiment. Ameliorating the environmental and testing conditions would also aid in normalizing zebrafish behaviors. This includes proper handling, the use of fewer and/or less stressful tests, and improving husbandry. If locomotor activity remains too low, extending the test for 6–12 more min may be a good practical solution, as it minimizes the initial anxiety and disinhibits zebrafish behavioral activity.

11.11. Zebrafish Display Aberrant Behavioral Phenotypes

Factors such as altered pain sensitivity, vestibular deficits, or motor/coordination impairments may nonspecifically alter animal behavior in a way that could be misinterpreted as altered anxiety phenotype. In addition, peculiar "rare" forms of zebrafish behavior may be present, and need a more careful in-depth behavioral assessment. For example, zebrafish treated with hallucinogenic or opioid drugs may exhibit trance-like passive swimming or epilepsy-like states that will confound analysis by video-tracking

software. To address this possibility and rule out all nonspecific factors, a careful examination of zebrafish neurological and sensory phenotypes is recommended.

12. Conclusion

Overall, video-tracking of zebrafish yields objective analysis of behavioral endpoints, and therefore provides researchers with an important tool for the investigation of anxiety and stress behavior in this animal model (**Figs. 1.2, 1.3,** and **1.4**). Representing a significant improvement over more subjective manual recordings (that are prone to human error), the video-tracking approach introduces the capability of observing additional behavioral endpoints not captured in manual recordings (**Table 1.1**). This research strategy allows an accurate and standardized measurement of anxiety-related behavior in zebrafish for sound data collection and analysis. Furthermore, such standardization promotes reproducibility in experimental design, strengthening the investigator's ability to draw valid conclusions from zebrafish study data and results.

Acknowledgments

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Chapter 2

Videograms: A Method for Repeatable Unbiased Quantitative Behavioral Analysis Without Scoring or Tracking

Russell C. Wyeth, Oliver R. Braubach, Alan Fine, and Roger P. Croll

Abstract

We present a method that complements both scoring by observers and automated tracking methods for quantifying behaviors. Based on standard motion enhancement, our algorithm converts a behavioral video recording into a single image ('videogram') that maps the spatial distribution of activity in the video sequence. This videogram can be used as a visual summary of activity and also as a direct, repeatable, and unbiased measure of animal activity. We describe the algorithm, and then use videograms to show acquisition of odorant-dependent place-conditioning in zebrafish trained in groups. We also demonstrate its potential for determining depth preferences and swimming speeds. This method generates activity measurements suitable for continuous variable statistics, and can be considered as an analysis alternative to behavioral tracking (over which it can have several advantages) for experiments not requiring exact trajectories.

Key words: Videogram, quantitative analysis, animal activity, place conditioning, depth preference, swimming speed.

1. Introduction

Quantitative analysis of animal behaviors is an important tool in zebrafish and other animal research (1, 2). Acquiring measurements from behavioral observation or video sequences has previously been based on manual scoring, e.g., (3–6) or tracking the behaviors, e.g., (7–12). Our goal here is to describe an alternative method for acquiring quantitative data that may be useful in behavioral experiments (with zebrafish or other animals).

A range of factors can be considered when choosing a behavioral analysis method. Scoring behaviors based on predetermined

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criteria creates quantitative data suitable for statistical analysis and thus objective assessment of behavioral responses to different treatments, e.g., (3–6). However, data from scoring are often categorical, and thus are unable to differentiate amongst subtle variations in behaviors, and also limit the range of applicable statistical tests. Moreover, scoring can be subject to observer bias and is often time-intensive. In particular, the time invested in scoring more than a few criteria is often prohibitive, and therefore restricts the range of metrics used to analyze behaviors. On the other hand, tracking behaving animals or their body parts creates excellent datasets that are both usable with continuous variable statistics and flexible with regard to analysis metrics, e.g., (9–13). However, tracking animals manually is especially laborious (pers. obs.), and automatic tracking systems require stringent image quality regulation (since a unique object needs to identified for tracking in each frame and mistakenly tracked objects can cause large deviations in tracks), are computationally intensive, and commercial packages are expensive. Moreover, many automated tracking systems cannot handle multiple animals if the possibility exists for their tracks to cross, although custom algorithms and software have been developed to overcome this problem, e.g., (14).

To complement these existing methods, we have developed an algorithm to reduce a video sequence into a single image (a "videogram") that measures the spatial arrangement of activity levels in the sequence. We employ standard motion enhancement, e.g., (7, 10, 15, 16) subtracting a background image from each video frame. The resulting images show lighter moving objects on a dark background. We then use a threshold to convert each to a binary image with white areas of activity in an otherwise black field (the subtracted background). However, rather than tracking the location of those white regions, we sum the images to create a spatial map of activity over the entire video sequence. The result is an image (the videogram) with lower intensity (darker) areas that had little or no activity during the video sequence, and higher intensity (lighter) areas that had more activity. This intermediate option for quantitative behavioral analysis provides repeatable, unbiased video analysis and yields continuous variable metrics without the complications of individual tracking. Furthermore, videograms can be used for analysis of either individuals or groups (that is activity of the group as a whole, not the activity of multiple individuals within a group). The method is computationally simple, can process far more frames than manual observations, and can be implemented in common image-processing packages (Matlab, ImageJ, Python, etc.). We describe here how to create a videogram from a behavioral video sequence, and offer optimization and troubleshooting tips. We then demonstrate its use by showing acquisition of

odorant-dependent place-conditioning in groups of adult male zebrafish, as well as brief examples of depth preference and swimming speed analyses.

2. Materials

2.1. Equipment

1. Digital video recording equipment.
2. A personal computer and image processing software.

2.2. Equipment Setup

The choice of camera and digital video recording equipment depends primarily on the experimental setup. Videograms can be created from any resolution video sequence recorded at any frame rate, with any (or no) video compression. The only requirement is a digital video file that captures the behavior of interest.

The algorithm described below can be implemented in any image-processing program that provides basic arithmetic image manipulation functions. In addition, software that allows the use of macros or programming will usually be highly advantageous (e.g., ImageJ, National Institutes of Health; Matlab, Mathworks, Inc.; Python, Python Software Foundation; etc.), although it could also be executed manually in programs such as Photoshop (Adobe Systems, Inc). In addition, a utility to convert a color video sequence to grayscale and to convert the video sequence to a series of images may be needed (e.g., VirtualDub, virtualdub.org; iMovie, Apple, Inc.).

3. Procedure

Videograms can be created from grayscale digital behavioral video sequences of one or more animals, measuring either individual or group activity, respectively (**Fig. 2.1**).

CAUTION: A high contrast source video sequence with no contaminating movements is important. Ideally, the animal(s) should be consistently darker or lighter than the background and they should be the only moving objects in the video sequence, although some deviations from this ideal are surmountable.

CAUTION: Videograms created from thousands of frames will likely need to be created using frame-by-frame processing rather than the simpler all-frames-at-once procedure presented here (see **Section 4.4** below).

The following steps create a videogram.

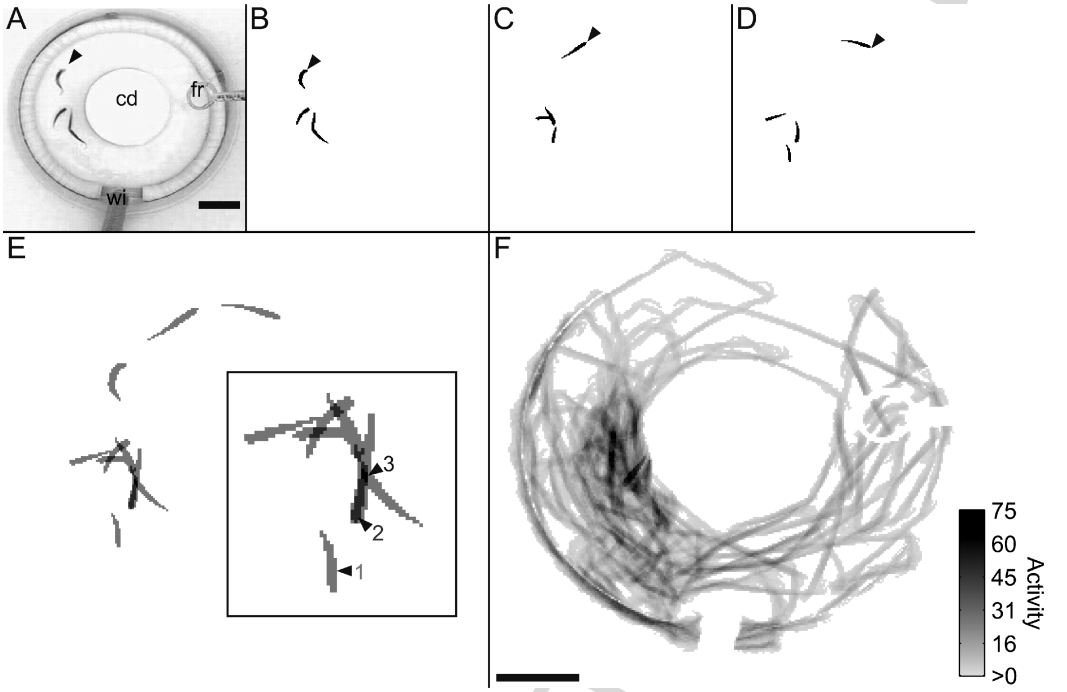


Fig. 2.1. Videogram creation. (**a**). A single frame from a video sequence of four zebrafish (one indicated, *arrowhead*) in an odor conditioning experiment (17). The camera is *above* a circular tank with a water inflow tube (wi), covered drain (cd), and a feeding ring (fr). (**b–d**). The same video frame and two subsequent frames (separated by 0.13 s) after background subtraction and application of a threshold, showing the inverted binary images of the four zebrafish (the same fish is indicated in each frame, *arrowhead*). *Black* pixels are indicative of activity at that location in that frame, since stationary fish would not be measured. (**e**). A videogram created by summing the frames in **b, c**, and **d**, with pixel intensities scaled to indicate activity levels. Inset shows how pixels occupied by a fish in just one frame (1) are 33% *gray*, in two frames (2) are 66% *gray*, and all three frames (3) are *black*. (**f**). A videogram of the entire video sequence, showing the distribution of activity in the tank. Activity scale: activity frequency over 30 s, sampled at 30 frames s^{-1}. Scale bars: 4 cm, shown in **a** for **a–d** and **f** for **e** and **f**.

NOTE: A demonstration of the procedure in ImageJ is available (*see* **Appendix**), as well as a more complex and versatile implementation in Matlab available upon request.

1. Convert the video sequence to a series of grayscale images, using a conversion utility if necessary. Uncompressed image formats are preferable since they do not blur contrast with compression. The video sequence is now a series of images, each with a rectangular array of pixels. Each pixel has an intensity representing its gray value, typically between 0 (black) and 255 (white), although greater bit-depth systems will also work. For example, an image of a zebrafish in a tank may have darker fish (pixel intensities ~50) swimming in front of a lighter background (pixel intensities ~200).

2. **CRITICAL STEP**: Ensure the moving animal of interest in the video sequence is lighter than the background. If the

animal is darker than the background (this is usually the case for zebrafish), invert all the images, reversing the grayscale.

3. Create a background image using one of three options:
   a. Option 1: use an image from a baseline portion of video sequence without any animals present (e.g., recorded before fish are introduced into the tank).
   b. Option 2: use an "absolute" mean image calculated from the entire behavioral video sequence. A subset of the frames can be used, provided the animal is not visible in the mean image.
   c. Option 3: use a "running" mean image calculated from a number of frames before and after the frame of interest.

   Any of these options can work successfully. Theoretically, a baseline image works best. However, practically an absolute mean image is the easiest to acquire, and a running mean image may be the only option if a dynamic background is present (*see* **Section 4**).
4. Create a series of subtraction images. Subtract the background image from each video frame image. Any regions of a video frame that are the same as the background will disappear (i.e., the pixel intensities are identical, and thus the subtracted image pixel intensities will be zero). Similarly, any regions darker (i.e., lower pixel intensities) will also disappear. Only regions of the video frame image that are lighter than the background image will have a pixel intensity greater than zero in the subtracted image. Thus, lighter moving objects (e.g., a swimming zebrafish in an inverted video sequence, *see* Step 2) will be the only objects visible in the subtracted images.
5. Create a series of binary images by applying a threshold to the subtraction images. Choose a threshold pixel intensity that separates the moving object of interest (e.g., the zebrafish) from any background noise. Importantly, the original video sequence must have enough contrast to consistently separate large fluctuations in pixel intensity caused by the animal, and small fluctuations in pixel intensity, created by the video camera and/or digitization process. The series of binary images now contain white regions with a pixel intensity of one, representing areas of activity (e.g., a swimming zebrafish) and black regions with a pixel intensity of zero, without activity.
6. Sum the series of binary images. The video sequence has now been converted to a single image, where the pixel intensity represents the number of frames during which activity occurred in that pixel. Black regions with zero pixel intensity show where no activity occurred in any of the binary

frames. Higher pixel intensity values show where more activity occurred (e.g., where the zebrafish swam more often).

Once a videogram is created, the pixel intensity represents the frequency of activity in that pixel's location over the entire video sequence. The videogram pixel intensity is equal to the number of frames for which the source video was higher than the threshold intensity, and thus provided only the moving animal is above threshold, the videogram pixel intensity measures how often the animal occupied that pixel location. If a baseline image without the animal present is used for subtraction (step 4), the pixel intensity measures occupancy. Alternatively, if a mean image is used for subtraction, the algorithm relies on motion (a motionless fish would produce a black videogram) and thus the pixel intensity measures activity (not occupancy). This occupancy or activity measurement is true whether a single animal or multiple animals were recorded in the original video sequence. In the latter case, the videogram simply represents the activity of the group of animals.

IMPORTANT: For display purposes, the pixel intensities will usually need to be normalized to a standard gray scale to avoid saturation. The videogram can then be used a qualitative demonstration of the spatial distribution of activity (**Fig. 2.1**). Conversion into a quantitative behavioral measure will depend on the source video sequence and the activity being measured. For example, if the video sequence shows a zebrafish in a tank, the mean depth occupied by the fish can be calculated by using all pixel intensities as weights for a weighted mean of the vertical pixel coordinates (**Fig. 2.2**). Alternatively, if the zebrafish are subjected to treatments that may attract them to a location in a tank, then the mean pixel intensity in that region is a direct measure of the animal's presence in that region (**Fig. 2.1**). These are just two examples, but the range of possibilities for such measures is limited only by what can be captured in a video sequence and the algebraic manipulation of pixel intensities and coordinates.

4. Optimization

4.1. Source Video Sequence

The quality of the source video sequence affects whether a videogram accurately measures activity. The resolution and compression algorithm used in the source video sequence are important only insofar as they affect whether or not the behavior is still visible in the video sequence. However, contrast between the animal and the background is paramount, since areas where the animal has similar gray values to the background cannot be analyzed.

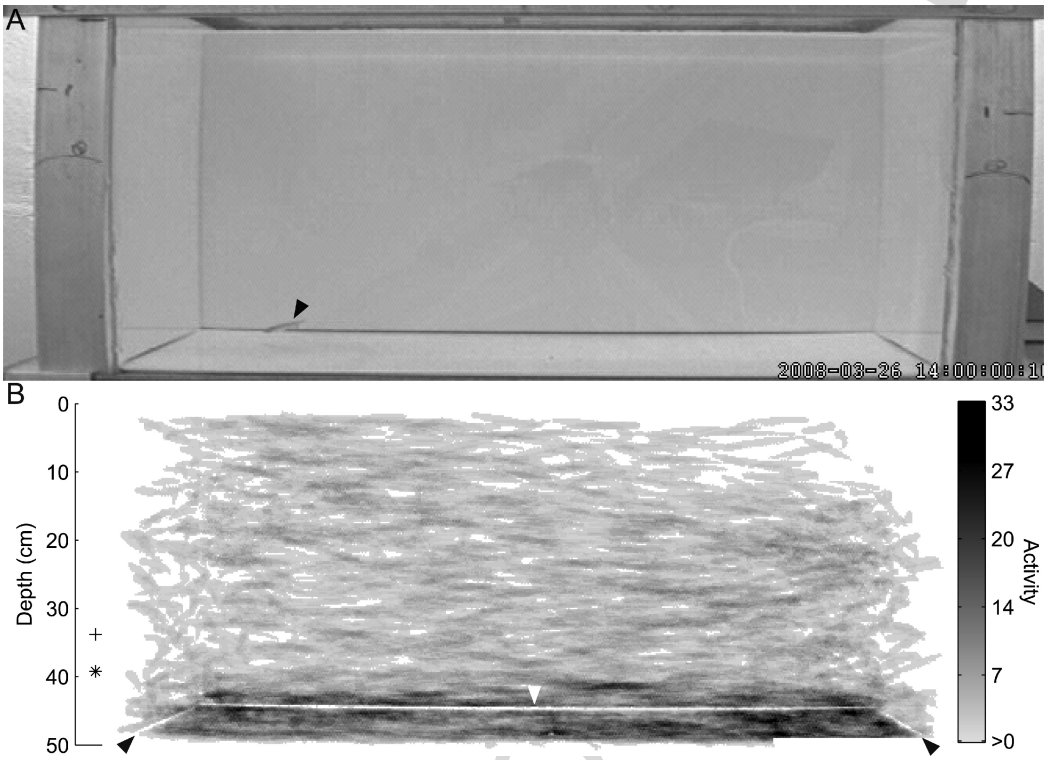


Fig. 2.2. A videogram used to measure depth preference of zebrafish. (**a**). A single frame from a video sequence of one zebrafish (*arrowhead*). (**b**). Videogram showing how the distribution of activity is concentrated toward the *bottom* of the tank, as expected for a zebrafish newly introduced to a tank. The distinct lines of missing activity (*arrowheads*) are due to the close match between the pixel intensities of the zebrafish and tank joint. These lines also emphasize the effect of parallax on a two-dimensional videogram, which conflates different depths in the three dimensional tank. Analysis of the video time stamp (*lower right*, **a**) was excluded using a region of interest. Activity scale: activity frequency over 1 h, sampled at 1 frame s^{-1}. Inset: mean (+) and median (∗) depth of the zebrafish calculated from the activity values (i.e., frequencies) and vertical pixel coordinates of the videogram.

Depending on the experiment, small regions of low contrast may be inconsequential (**Fig. 2.2**), as they may only be a small proportion of the recorded activity (e.g., if a zebrafish swims in front of tank joint and "disappears" in a still video frame). However, careful choice of both lighting and background materials (e.g., lining three sides and the bottom of a zebrafish tank with white Plexiglas) will greatly improve consistent detection of activity. If color provides the best contrast, then the algorithm can be modified to use red, green, blue, hue, or saturation values in place of grayscale intensities in Step 1. In addition, the animals must be the only moving objects in the video sequence. Any movements created by the experimenter, abrupt changes in background, reflections (e.g., zebrafish reflected from underside of the water surface) cannot be distinguished from animal activity. Cameras and lights should therefore be placed to avoid contamination by extraneous movements.

Videograms, similar to tracking, are subject to the disadvantages of using a two-dimensional view of a three-dimensional behavior. For example, when tracking zebrafish depth preferences, parallax causes different tank depths to appear at the same position in both single video frames and videograms (**Fig. 2.2**). These problems are common to all video analysis methods, and can be eliminated by employing multiple cameras or mirrors or mitigated by choosing camera positions and lenses that minimize parallax.

4.2. Video Frame Rates and Durations

Choosing frame rates and durations depend on the behavior under analysis. Faster frame rates create track shapes that show entire movements. For example, in zebrafish, a faster swimming animal will create a longer, but less intense track of non-zero pixels in a videogram than a slower moving animal (**Fig. 2.3**). The intensity and track area values can then be used as measures of swimming speed without ever tracking the fish. Alternatively, if the videogram itself is then converted into a binary image, standard analysis methods can be used to calculate the dimensions of such a region, and thus swim speed (or other locomotory

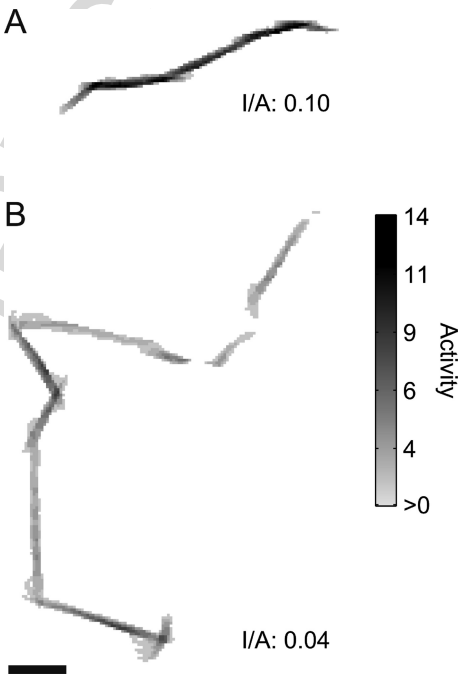


Fig. 2.3. Two videograms distinguish slow and fast swimming zebrafish. **a**. A slowly swimming fish creates a relatively short movement trace with high pixel intensities. **b**. A fast swimming fish creates a relatively long movement trace with gaps and low pixel intensities. An intensity to area ratio quantifies the difference between slow and fast swimming fish (I/A = summed intensities of all non-zero pixels/number of non-zero pixels, activity pixel^{-1}). Activity scale: activity frequency over 2 s, sampled at 30 frame s^{-1}. Scale bar: 2 cm.

variables such as the acuteness of turns) can be directly calculated, still without any tracking. Slower frame rates sample the activity at intervals. If these are used over longer duration video sequences, then the general location of activity will be depicted by the videogram (**Figs. 2.1** and **2.2**).

4.3. Region of Interest (ROI)

An ROI can be used to exclude certain areas of the video sequence, or alternatively, include only certain areas. For example, video time stamps and extraneous motion around the periphery can be excluded using an ROI (**Fig. 2.2**). Alternatively, ROIs can restrict analysis to the areas where the behaviors of interest occur or create multiple individual videograms for multiple animals in a single frame.

4.4. Processing Speed

Three primary techniques can reduce processing times. An ROI can be used to crop the pixel dimensions of every image in the series, reducing the total number of pixels processed. Frame-by-frame processing can also be beneficial or essential for large numbers of frames that cannot be simultaneously loaded into computer memory. Rather than applying each step of the algorithm to all frames before moving on to the next step, a running summed binary image is kept as the steps are applied to each frame in sequence. This enhances speed because it avoids loading all images into computer memory simultaneously, and also allows examination of the effect of different settings without processing all frames. Furthermore, since single frames can usually be processed entirely in computer memory, it can also be used to reduce the number of files written to hard disk, often a strong contributor to processing time (although this eliminates the possibility of reviewing the various steps of the algorithm and will thus reduce troubleshooting options). Finally, longer durations and higher frame rates increase processing time, and if these can be reduced without compromising the capture of the behavior, then shorter calculations are possible. For example, depth preference measurements at 30 frames s^{-1} generate 108,000 samples h^{-1}, yet provide similar depth information (data not shown) as a videogram based on 3,600 samples generated from at 1 frame s^{-1} (**Fig. 2.2**).

4.5. Comparing and Combining Videograms

Comparing and combining videograms add an additional requirement that the individual videograms be scaled similarly. This accounts for both variations in video sequence duration or frame rate and also the possibility of dropped frames during the digitization process. Dividing pixel intensities by the number of frames in the source video sequence standardizes the videograms to activity $frame^{-1}$, and allowing comparison amongst all video sequences recorded using the same video setup. Standardized videograms can also be averaged to examine activity pooled from multiple video sequences (**Fig. 2.4**). In this case, provided the video

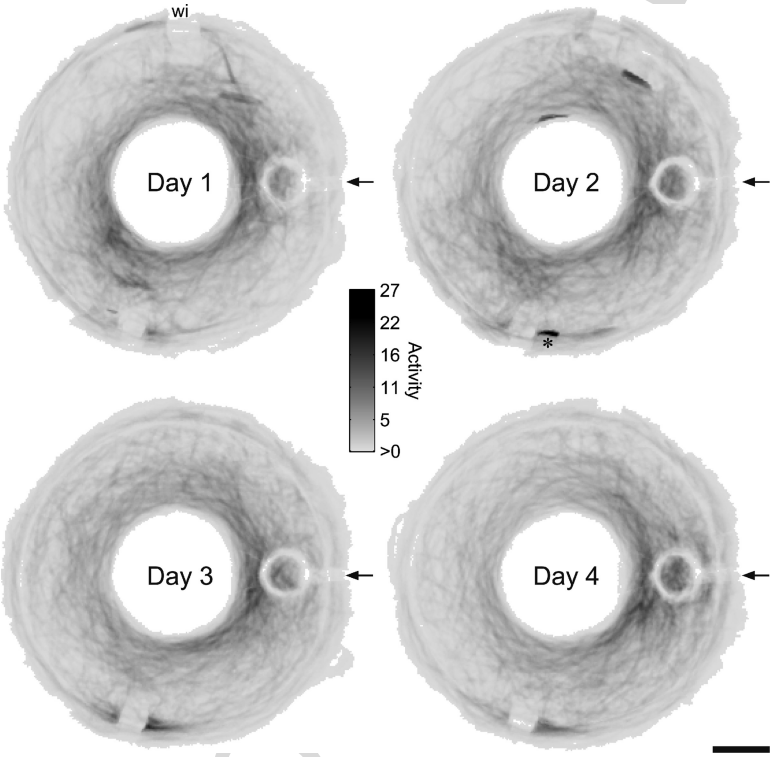


Fig. 2.4. Averaged videograms show the acquisition of an odorant-dependent place preference by zebrafish trained in groups. Six groups of 4 fish were trained over 4 days to associate an odor with food provided inside a feeding ring (*arrows*) see (17) for details. Videograms were created from video sequences showing the fish behaviors after odor presentation (conditioned stimulus), but before food reward administration (unconditioned stimulus). Videograms from 3 trials per day for each group of fish were mapped onto a common coordinate system with the same feeding ring location, and then averaged across all six groups. The concentration of activity near the feeding ring after odor presentation increased each day (as opposed to the opposite side of the tank, which showed decreasing activity). Distinct areas of reduced activity are due to water inflow tubes with varying locations across the six training groups (one indicated, wi). Distinct areas of high activity, particularly on day 2 (∗) are a result of fish 'hiding' in certain locations of the tank due to dominance behaviors in one group. Activity scale: average activity frequency over 30 s, sampled at 30 frames s^{-1}. Scale bar: 4 cm.

sequences can be registered by mapping to a common coordinate system, the video sequences need not be taken with same camera nor even be of the same scene.

5. Trouble shooting

The easiest method to confirm a videogram accurately depicts animal activity alone is to create a video sequence from the series of binary images (Step 5). Watching the source video sequence

followed by the binary video sequence will highlight any anomalous areas that are measured as activity but are not created by the animal(s). In the case of zebrafish, this can often be reflections, air bubbles, the water meniscus, camera or tank movements caused by clumsy experimenters, or simply random noise created by pixel fluctuations. If these cannot be eliminated by optimizing the source video sequence, several processing methods can be used to filter them from the videogram. Digital image filters (e.g., a median filter) can be used to remove random white noise from subtraction images (Step 4). Adaptive thresholds, adjusted based on the total pixel intensity of an image can allow creation of consistent binary images (Step 5) despite fluctuations in lighting (for example, if lighting alternates between visible and infrared illumination). Alternatively, standard image processing methods allow statistics (dimensions, area, concavity, etc.) to be gathered on all objects in a binary image (an object is a contiguous area of white pixels). If spurious activity regions have consistently different shapes from those generated by the moving animals, then these object statistics can be used to select and erase them from the binary image series (16).

Dynamic backgrounds are another factor that can compromise the accuracy of a videogram. However, careful choice of video frame rate and the frames used to calculate a mean image as a background image (Step 3) used in image subtraction (Step 4) can circumvent this problem. As long as the background changes more slowly than the animals move, then a mean image that is calculated relative to the frame being processed should be able to highlight animal activity alone. The key is to select frames at intervals both before and after the frame being processed such that the animal's activity is blurred into the background, while that averaged background still resembles the background in the frame being processed (for example, every 5th frame from the 25th frame preceding frame to the 25th frame following). Alternatively, if there are slight changes in background and foreground between video sequences (particularly as consequence of a different camera position), image registration can be used to transform the videograms to a common map, allowing comparisons to be made accurately.

Finally, if the contrast of the moving animal is dynamic, with either higher or lower pixel intensity than the background, then clipping of activity can occur. For example, if calculations are designed to detect a dark zebrafish moving over a light background, then no activity will be detected if the fish moves to an area where it appears lighter than the background. In this case, an absolute value subtraction image can be created (or the sum of two subtraction images: the frame minus the background and the inverted frame minus the background). This will enhance activity with either higher or lower pixel intensity in the source

video sequence, but will be unable to enhance activity in regions where the contrast is in transition. Moreover, the subtraction images will be inherently noisier, and increasing the likelihood post-processing will be needed.

6. Anticipated Results

Videograms can be used for both qualitative observations of large video data sets as well as quantitative analysis. For example, we implemented our videogram algorithm in Matlab (source code available upon request) to examine the acquisition of odorant-dependent place-conditioning during group training of zebrafish (**Fig. 2.1**). Braubach et al. (17) trained groups of fish to associate an odor (conditioned stimulus) with a food reward provided inside a feeding ring on one side of a circular tank (unconditioned stimulus). After training, individual animals spent more time near the feeding ring when odor stimuli were applied, and thus had developed an odorant-dependent place preference. We therefore reasoned that the training video sequences should show the progressive acquisition of this place preference, without the need for tracking individual fish within the groups. To examine the change in fish behavior, we created an averaged videogram for each day of training (**Fig. 2.4**). Combining data from three 30 s training trials per day for six groups of four fish, each averaged videogram provides an unbiased objective analysis of 16,200 video frames. They demonstrate how on the first day the fish do not concentrate their activity near the feeding ring when exposed to the conditioned odorant. However, on each subsequent day the fish activity distribution is increasingly biased toward the feeding ring. Although this trend is not as consistent when measuring the total activity within 6 cm of the ring (**Fig. 2.5**), if activity is measured as a proportion of the total over the entire tank (a better measure of the any place preference, in our view), a linear regression over the conditioning period showed a significantly ($R^2 = 0.18$, $F_{1,22} = 5.1$, $P = 0.035$) increasing proportion of activity that occurred within a 6 cm radius of the feeding ring center (**Fig. 2.6**). Thus, we are able to use videograms to show the changes in behaviors captured in video sequences from multiple cameras on multiple days, and also to find quantitative evidence that that odorant-dependent conditioning can occur in groups of zebrafish trained together.

Videograms are versatile and can be used with almost any behavioral video sequence with reasonably consistent contrast. Both the location and level of activity in the videogram can be measured, allowing the calculation of spatial preferences and other behavioral parameters. For example, a slow swimming

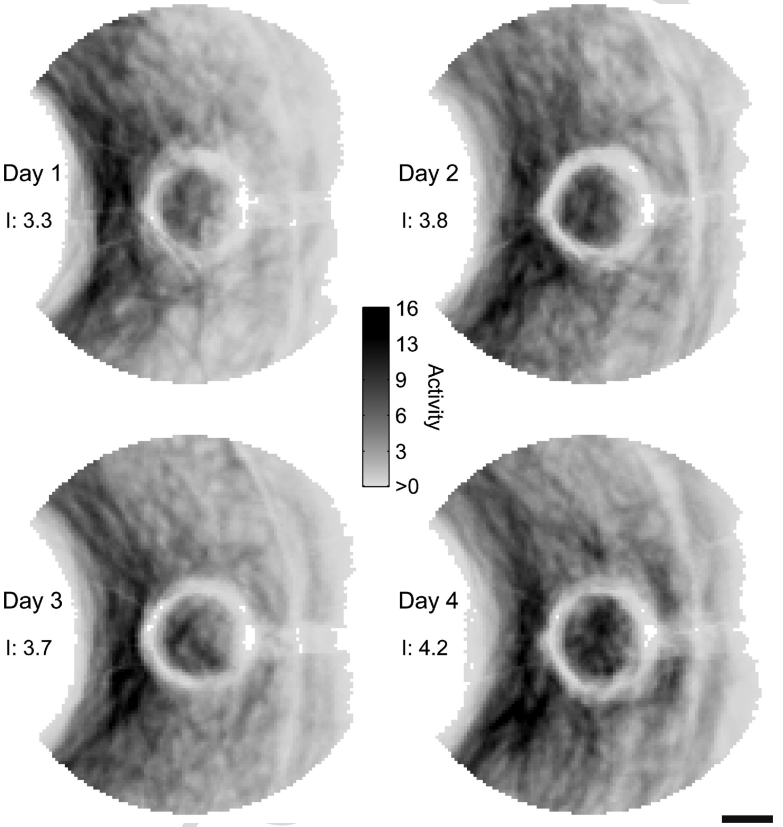


Fig. 2.5. Magnified averaged videograms (*see* **Fig. 2.4**) showing only the analysis regions around the feeding rings. Averaged across the six groups of 4 zebrafish, the total activity within 6 cm of the centre of the ring after odor presentation increased between days 1 and 2, showed a small decrease between days 2 and 3, and increased again between days 3 and 4 (I, summed mean activity pixel^{-1}). Activity scale: average activity frequency over 30 s, sampled at 30 frames s^{-1}. Scale bar: 2 cm.

zebrafish creates a short, bright videogram whereas a fast swimming zebrafish creates a long dim videogram (the binary images of the slow swimming fish overlap more between frames, and thus the videogram trace has a high intensity but a smaller area after summation, whereas the fast swimming fish has less overlap and thus lower intensity and larger area). Accordingly, an intensity: area ratio provides a convenient metric for distinguishing slow versus fast swimming fish (**Fig. 2.3**). Based on both location and intensity measures, we have used videograms to measure depth preferences during tank acclimation (**Fig. 2.2**), analysis of swimming speeds or trajectories (**Fig. 2.3**), startle responses (Stoyek and Croll, in prep.), or analysis of larval olfactory behaviors (Braubach, Fine and Croll, in prep.). Yet other behavioral parameters can be measured based on further analysis of the videogram. Since the videogram is an image showing activity levels, a threshold can be applied to convert it into a binary image, with a black background of low activity (below the threshold) and a white

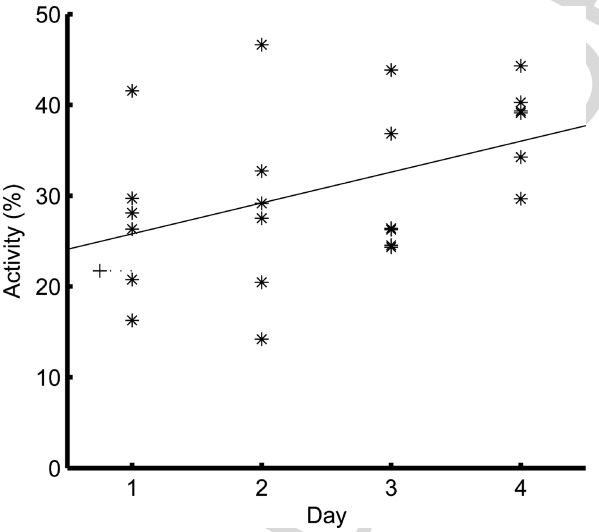


Fig. 2.6. Activity calculated from videograms shows the acquisition of odorant-dependent place-conditioning by zebrafish trained in groups. Six groups of 4 fish were trained separately over 4 days to associate an odor with food provided inside a feeding ring, see (17) for details. The sum of activity within 6 cm of the ring centre as a proportion of all activity in the tank was averaged from videograms of 3 trials per day for each group of fish. Initially, activity near the ring was similar from what would be expected by chance (+ indicates baseline activity before training began, averaged from single videograms of each group of fish). However, a linear regression over time (*solid line*, $P = 0.035$) shows a significant increase in activity close to the ring indicative of place-conditioning.

region of high activity (above the threshold). Commonly available image analysis methods can quantify the shape of the high activity region (see the documentation for ImageJ, Matlab Image Processing Toolbox, etc.), enabling measurements of speed (e.g., the white region feret/duration of video sequence), turn angle (difference between the angles of the major axes of two ellipses fit to two white regions from sequential videograms created just before and after a turn), tortuosity (aspect ratio of an ellipse fit to the region), etc. Furthermore, videograms can be used for analysis of other types of experiments as well. Physiological analysis of breathing movements, eye movements, or any other movement that can be captured on video sequence with consistent contrast can be measured with a videogram. Finally, if color figures are an option, we have found an overlay of a pseudocolored videogram on top of the background image from the video sequence to be a striking in-context demonstration of the activity distribution (8).

The activity patterns shown by videograms are similar but not identical to position traces created by tracking algorithms. A videogram shows the average activity distribution of the animal(s) over the video sequence, whereas a behavioral track is a continuous series of individual locations. Thus, for long tracks where the animal(s) repeatedly occupy the same location and the

track points become crowded and overlap, a videogram is better at showing the relative distribution of activity. (Note that tracking data could be converted to an image very similar to a videogram by mapping location frequencies to pixel intensity; however, this makes tracking redundant.) Moreover, errors in isolating the moving animal during the image processing steps have less of an effect on videograms (depicting averaged data) than tracks (depicting unitary data points).

This difference in error susceptibility and several other factors make the image quality requirements for videograms less stringent than for tracking in video sequences. Both videograms and tracking use background subtraction followed by application of a threshold to create a binary image, and thus both methods require consistent contrast and brightness. However, tracking algorithms must identify a single white region in the binary image created from each frame, requiring absolutely consistent pixel intensity contrast or alternatively an algorithm that handles two possibilities: (1) the animal "disappears" below threshold, and thus no white region is present and the frame must be skipped; or (2) the animal is represented by multiple white regions created by contrast fluctuations across the animal, and thus a filter must select one region or combine the multiple regions for successful tracking. Furthermore, extraneous white regions in the binary image (those not representing the animal) must be avoided entirely or filtered from each frame (by position, size, shape, etc.) for tracking to succeed. In contrast, the algorithm for videogram calculation requires no modification to handle frames where the animal "disappears;" and provided these are infrequent, the videogram will still accurately represent the spatial distribution of activity (the benefit of showing averaged data). Videogram calculation is also unaffected by multiple white regions due to pixel intensity fluctuations, and can still accurately represent animal's activity in a video sequence without filtering such fluctuations. Similarly, extraneous white regions can be rendered negligible by averaging sufficient frame numbers without extraneous regions, or they can be filtered from the final videogram (not necessarily every frame). Thus, the image quality requirements for consistent brightness and contrast, although still present, are considerably lower for creating videograms than tracking animals. Moreover, the complexity of the algorithm (and the programming code for automated analysis) is lower for videograms than tracking. Commercial software packages with tracking algorithms typically have a number of algorithms to handle contrast inconsistencies, but we suggest researchers requiring more economical options, custom analyses, or integration with other experimental requirements, and thus coding their own software, should consider the use of videograms.

In summary, we suggest videograms are a useful option for behavioral video analysis to be considered along with scoring and

tracking. Once the algorithm is optimized for a particular experiment and confirmed through pilot video sequences to accurately capture the activity of interest, videogram creation can be completely automated in an unbiased and repeatable fashion. This can allow both more extensive and more accurate analysis than scoring by observers. Videogram measurements are thus comparable to tracking data. Yet tracking requires more stringent contrast control since mistakes in tracking can result in large path deviations, whereas similar rare events have little effect on videograms calculated from many frames. Moreover, since most tracking algorithms rely on binary images to identify the location of animals being tracked, both videograms and tracking can be accomplished with considerable overlap in image processing. Thus, videograms can be used for both qualitative observation and quantitative measurement of behavioral video sequences, and complement either scoring or tracking of behaviors in experiments.

7. Appendix: Using ImageJ to Create a Videogram

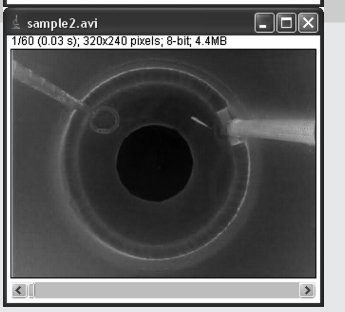
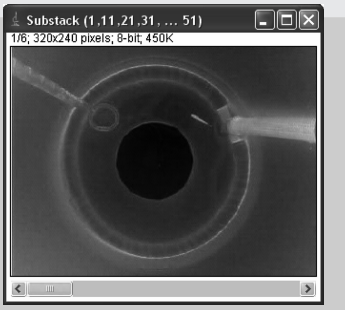
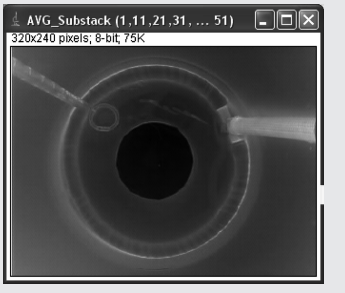
This document outlines a step-by-step procedure to produce a videogram from a short sample movie.

7.1. Requirements/ Preparation

1. **ImageJ**
   The procedure uses the MacBiophotonics ImageJ release, which bundles a number of necessary plugins (AVI Reader, Substack Maker, Handle Extra File Types) http://www.macbiophotonics.ca/imagej/
2. **Sample video**
   The procedure relies on the movie being opened directly in ImageJ. This only works if the movie is uncompressed. Therefore, do any *one* of the following:
   - Use an uncompressed AVI movie and load it into ImageJ using the AVI Reader plugin http://rsbweb.nih.gov/ij/plugins/avi-reader.html
   - Use a compressed movie and convert it to an uncompressed movie using another video processing program, and use the AVI Reader plugin.
   - Use a compressed movie and convert it to a series of uncompressed (TIFF, TARGA, BMP, etc.) images using another video-processing program, and then use the File: Import: Image Sequence. . . command in ImageJ to create a stack of grayscale images from the series of image files.

   The sample movie used in this example is available: http://people.stfx.ca/rwyeth/vidsimages.html or contact Russell Wyeth rwyeth@stfx.ca

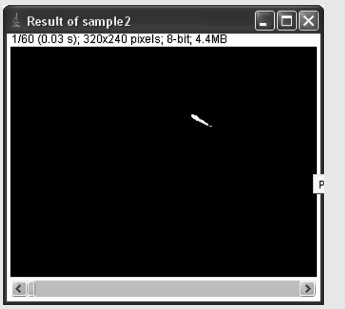
ImageJ commands to convert the uncompressed video "sample2.avi" into a videogram

Menu command in ImageJ *(v1.42 l, MacBiophotonics release)*	Image Window Result
1. File: Open browse and select "**sample2.avi**" **Open** *Only uncompressed AVI files can be opened by ImageJ.* First Frame: **1** Last Frame: **60** ☐ Use Virtual Stack ☑ Convert to Grayscale ☐ Flip Vertical **OK**	
2. Edit: Invert Process all 60 images? There is no undo if you select "Yes" **Yes**	
3. Plugins: Stacks – Reducing: Substack Maker Enter either range (e.g. 2–14) or a list (e.g., 7,9,25,27): **1,11,21,31,41,51** **OK** *This stack will be used to create the mean image.*	
4. Image: Stacks: Z Project. . . Start slice: **1**; Stop slice: **6** Projection Type: **Average Intensity** **OK** *This creates a poor mean image, with a considerable 'shadow' of the fish's motion, yet still suffices to demonstrate the method. A longer video providing more widely spaced frames (selected in step 3) would produce a mean image with little trace of the fish.*	

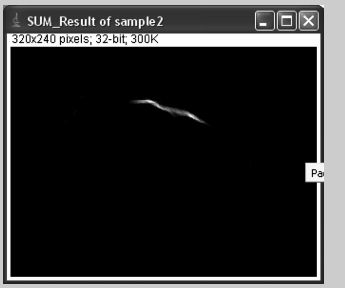
continued

5. Process: Image Calculator...
   Image 1: **Sample2.avi**
   Operation: **Subtract**
   Image 2: **AVG_Substack(1,11,21,31...51)**
   ☑ Create New Window
   ☐ 32 bit (float) Result
   OK
   Process all 60 images? ? There is no undo if you select "Yes"
   Yes

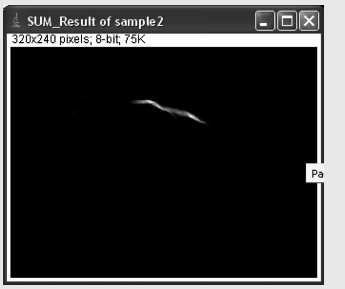
6. Image: Adjust: Threshold...
   [threshold minimum slider, top]: **20**
   [threshold maximum slider, middle]: **255**
   [threshold display, bottom]: **Red**
   Apply
   Convert all images in stack to binary?
   ☐ Calculate Threshold for Each Image
   ☑ Black Background
   OK



7. Image: Stacks: Z Project...
   Start slice: **1**;
   Stop slice: **60**
   Projection Type: **Sum Slices**
   OK



8. Image:Type:8 bit



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Chapter 3

Automated Imaging of Avoidance Behavior in Larval Zebrafish

Ruth M. Colwill and Robbert Creton

Abstract

This protocol describes the construction of an automated imaging system and two assays for measuring avoidance behaviors in larval zebrafish. The first assay, called the "bouncing ball assay," measures the response of larvae to a threatening stimulus displayed on an LCD screen. The second assay, called the "two-fish assay," measures avoidance behavior of two siblings in a multiwell plate. The assays are robust and can easily be adapted for medium- to high-throughput applications.

Key words: Automated imaging, avoidance behavior, larvae, bouncing ball assay, two-fish assay, ImageJ.

1. Introduction

Zebrafish larvae are ideally suited for large-scale analyses of behaviour (1–4). A modest colony of fish can produce hundreds of embryos on a daily basis and the embryos quickly develop into free-swimming larvae (5, 6). Automated imaging systems have been developed to monitor the activity of zebrafish larvae in 96-well plates (7–10). However, it remains challenging to analyze behaviors other than activity in high-throughput applications. The current protocol describes a novel high-resolution imaging system that was developed for measuring asymmetric behavior, predator avoidance, learning, and social interactions (11). The high-resolution imaging system is unique in its ability to measure both the location and the orientation of zebrafish larvae in multiwell plates. A variety of visual stimuli can be created and

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presented to the larvae on an LCD screen. Its programming flexibility regarding visual displays allows one to investigate the developmental trajectory of more complex aspects of learned behavior, including pattern discrimination, scene analysis, and stimulus preference.

This protocol provides information on how to build the imaging system and describes two assays for measuring avoidance behaviors: the 'bouncing ball assay' and the 'two-fish assay'. The bouncing ball is displayed on an LCD screen and may mimic the shadow of a large predator. Larvae quickly swim away from the bouncing ball, and then turn around to face the threatening stimulus from a distance. In nature, these responses may help the larvae to avoid predators and to see when a predator attacks. In the two-fish assay, two larvae are placed together in each well of a 12-well plate. The larvae prefer to stay far apart, which is surprising since adult zebrafish are social animals that prefer to swim in shoals. Possibly, larvae need to spread out in nature to avoid patch-foraging predators or to minimize competition for food. The two assays for measuring avoidance behavior can be used to identify individuals with varying degrees of boldness. Furthermore, excessive avoidance behaviors may indicate a predisposition for anxiety or fear and could provide new insights into the causes and treatments of human anxiety disorders.

2. Materials

2.1. Reagents

1. Instant Ocean (Aquatic Eco-systems, Inc, Apopka, Florida, Cat no. IS160)
2. Methylene blue (Sigma-Aldrich, St. Louis, MO, Cat no. M44907-100G)
3. Agarose (Sigma-Aldrich, St. Louis, MO, Cat no. A5093-100G)
4. Deionized water

2.2. Equipment

1. Tank with zebrafish, *Danio rerio* (Carolina Biological, Burlington, North Carolina)
2. Breeder tank, 2L (Aquatic Habitats, Apopka Florida)
3. Transfer pipettes (Fisher Scientific, Pittsburgh, Pennsylvania, Cat no. 13-711-9AM)
4. Large culture dishes for embryo culture (Fisher Scientific Cat no. 08-772-32; Corning no. 430591)
5. Large culture dishes for imaging (Fisher Scientific Cat no. 08-757-12)

6. Flat bottom 12-well plates (Fisher Scientific Cat no. 07-200-81; Corning no. 3512)
7. Shell vial (Electon Microscopy Sciences, Hatfield Pennsylvania, no. 72631-10).
8. Benchtop incubator set at 28.5°C (e.g., Fisher Scientific/Carolina Biological)
9. Cabinet/single tier wood locker (55 cm W × 35 cm D ×180 cm H)
10. Ultrathin light box (Electron Microscopy Sciences, Hatfield Pennsylvania, Cat no. 71649-5A)
11. Glass 5 gallon fish tank (Carolina Biological, Burlington, North Carolina)
12. Canon PowerShot SX110 IS digital camera (Tristate Camera, New York, NY). Note: some of the newer PowerShot SX cameras may not include remote capture capabilities (check with vendor). An alternative camera with remote capture is the Canon EOS Rebel T1i with an EF-S 55-250 lens and an AC adapter kit.
13. Power supply for Canon camera (Tristate Camera, New York, NY, Cat no. ACMV)
14. USB extension cord (at local computer store)
15. Imaging computer (e.g., Dell Optiplex, 3 GHz, 3 GB RAM, at local computer store)
16. Mini-laptop (Acer Aspire One, at local computer store)
17. Translucent sheet of plastic, Oxford 04491 (Office World, Inc., Eugene, Oregon)
18. PowerPoint software (at local computer store)
19. Microsoft Excel software (at local computer store)

2.3. Reagents Setup

Embryos are cultured in 'egg water'. Prepare egg water by adding 0.48 gram of instant ocean to 8 L deionized water (60 mg/L final concentration). This low concentration of salt mimics a fresh water environment, while avoiding copper and chlorine in the tap water. Add 0.2 ml of a 1% w/v methylene blue stock as a mold inhibitor (0.25 mg/L final).

2.4. Equipment Setup

The imaging system is constructed in a tall wooden cabinet, and can be set up in an upright configuration for the bouncing ball assay or an inverted configuration for the two-fish assay (**Fig. 3.1**). It is relatively easy to switch between the two configurations (11).

2.4.1. Upright Configuration

The upright configuration of the imaging system is shown in **Fig. 3.1A**. To build this system, take a tall wooden cabinet or

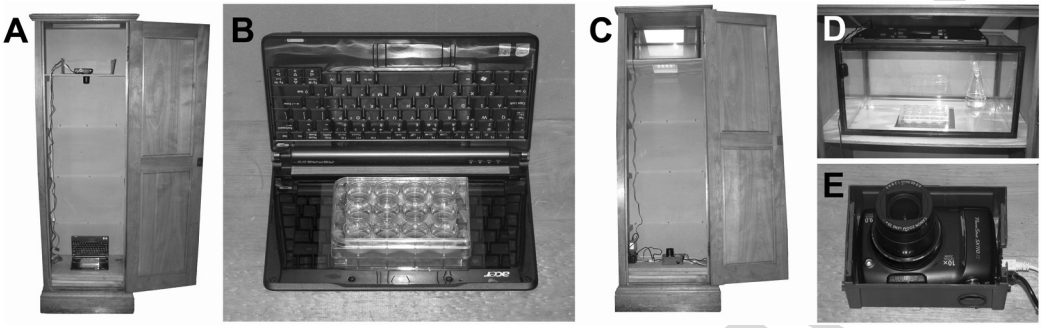

Fig. 3.1. The zebrafish imaging system. (**a**) Upright configuration with the high-resolution camera on the top shelf. (**b**) A mini-laptop is used for showing PowerPoint presentations to the larvae. (**c**) Inverted configuration with the camera on the bottom shelf. (**d**) Zebrafish larvae are placed in the imaging chamber, consisting of a glass fish tank on its side with a thin lightbox on top. (**e**) The digital camera has a 9 megapixel resolution and a 10× optical zoom. The distance between the camera and the larvae is 120 cm. When the camera is too close to the multiwell plate, the outer wells of a multiwell plate are imaged under a steep angle and the larvae may be obscured by shadows and blind spots (adapted from Creton (11)).

single tier locker (180 cm high) and remove all shelves, except for the top shelf. The top shelf should be positioned 120 cm above the bottom of the cabinet. Cut a 5 cm hole in the top shelf and place the Canon camera on this shelf, aligning the camera's lens with the 5 cm hole. While the camera can be operated on its batteries, an AC power adaptor is needed for remote capture. Install Canon's ZoomBrowser EX 6.1 software (included with the camera) on the imaging computer and connect the camera to the computer with a USB extension cord. Place the mini-laptop on the bottom of the cabinet. The LCD screen of the laptop needs to be oriented horizontally (**Fig. 3.1B**). A thin translucent diffuser (Oxford 04491) is placed on the LCD screen to blur the pixels of the screen. Culture dishes with zebrafish larvae will sit on top of the translucent diffuser.

2.4.2. The Bouncing Ball Stimulus

To create a bouncing ball stimulus, open PowerPoint, draw the perimeter of a large culture dish (9 cm diameter), and create a black disc with a 2.5 cm diameter just outside the culture dish (top left). Right-click on the disc and select: custom animation, add effect, motion path, right. The bouncing ball should start on the left and end on the right, just outside the dish. Double-click on the motion path, and select: auto-reverse (no smooth start or end), timing, speed, 3 s (slow), repeat, until end of slide. The bouncing ball will now move up and down in the top half of the dish and will be located adjacent to the culture dish once every 3 s (**Fig. 3.2A**).

2.4.3. Inverted Configuration

The inverted configuration of the imaging system is shown in **Fig. 3.1C–E**. To construct the zebrafish imaging system in this configuration, take one of the extra cabinet shelves, cut a 16×16 cm hole in the center, and use the board as the top shelf in the

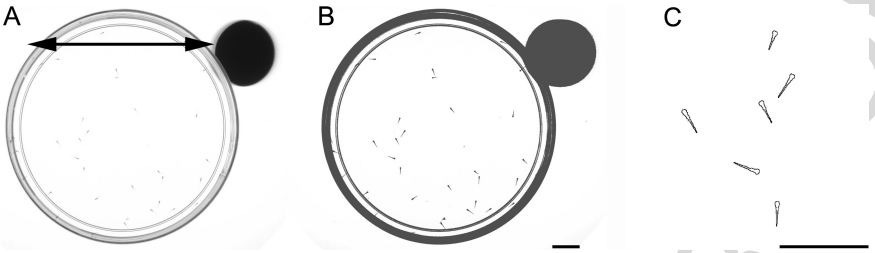

Fig. 3.2. The bouncing ball assay. (**a**) The majority of zebrafish larvae swim away from the bouncing ball. (**b**) Larvae are separated from the background using a threshold for dark objects. (**c**) Particles that are smaller or larger than the larvae are filtered out using the "analyze particle" feature in ImageJ. The larvae's centroids and center of mass are exported to MS Excel as a series of X,Y coordinates. Larvae are 6 days old. Scale bar = 1 cm.

cabinet. Place a 5-gallon glass fish tank on top of the shelf. The fish tank should lie on its side to provide easy access to the inside of the tank. The culture dishes will sit inside the tank, and will be imaged from below. Put an ultra-thin light box on top of the fish tank as a light source. Place the camera on the bottom of the cabinet, 120 cm below the glass tank. Connect the camera as described above.

3. Procedure

3.1. Zebrafish Larvae

1) Maintain a population of adult zebrafish, *Danio rerio*, at 26–28°C on a 14 h light, 10 h dark cycle. A mixed population of males and females will spawn daily, typically within an hour after the tank lights turn on. The fertilized eggs drop to the bottom of the tank. To prevent the fish from eating the eggs, add a layer of small marbles (1 cm diameter) on the bottom of the tank or use a collection tray with a 2 mm mesh screen (e.g., Aquatic Habitats, 2 L breeder tanks). Detailed protocols for breeding zebrafish are available in the zebrafish book, which is available online at http://zfin.org/ (12).
2) Embryos are grown in an incubator at 28.5°C on a 14 h light –10 h dark cycle until 6 or 7 days post fertilization (dpf). Grow approximately 30 embryos in a 9 cm culture dish containing 50 mL egg water. Maintain a high humidity level (e.g., by placing a beaker of deionized water in the incubator) to reduce evaporation of the egg water from the culture dishes. Check the culture dishes daily and remove dead embryos with a transfer pipette (cut the pipette tip to increase the diameter of the opening). Dead embryos are easily recognized as they are more opaque than live embryos. Embryos will hatch from their chorion between 2 and 3 dpf. The free-swimming larvae use the nutrients in their yolk during the first week of development.

3.2. The Bouncing Ball Assay

1) At 6 dpf, transfer 30 larvae to a new 9 cm culture dish, 20 min before the imaging experiment. Fisher brand culture dishes (08-757-12) are good for imaging, as these dishes do not have the inner 'ring' shown in **Fig. 3.2A,B**.
2) Set the imaging system up in the upright configuration (**Fig. 3.1A**). Turn the Canon camera on and open "Remote Capture" in the ZoomBrowser software. Select the following settings in the Remote Capture Task Window: maximum optical zoom (10× optical, 1× digital), image quality = medium 1 normal (2816×2112 pixels), white balance = fluorescent, iso speed = 200, aperture (Av) = 5.6, and exposure time (Tv) = 1/10 s.
3) Start the PowerPoint presentation with the bouncing ball on the mini-laptop, with the laptop's LCD screen in a horizontal position. Cover the screen with a translucent sheet of plastic. Then place the dish with 6 dpf larvae on top of the plastic sheet. The LCD screen warms the culture dish to approximately 28°C. To avoid condensation, leave the culture dish uncovered during imaging.
4) Click on the Remote Capture "refresh button" to focus the camera and go to 'shooting' to activate the interval shooting mode. Set the image interval at 63 s and the number of images at 40. Click on 'start recording' when the bouncing ball is located next to the culture dish. Subsequent images should show the ball just outside the dish, alternating between the left and right side of the dish (**Fig. 3.2**).
5) Acquired images are saved as 0.6 MB JPEGs for further analysis.

• **TIMING** The bouncing ball assay takes approximately 1 h. The assay may be adapted for 6- or 12-well plates for medium- to high-throughput applications.

3.3. The Two-Fish Assay

1) Create imaging chambers in a 12-well plate by adding 1.5 mL of melted agarose (1% w/v in egg water) in each of the wells. Let the agarose harden, and punch a hole in the agarose using a 14-mm shell vial (Electon Microscopy Sciences). The holes should be centered to avoid shadows along the edges of the well. To punch the hole exactly in the center of the well, wrap tape around the glass vial until it matches the inner diameter of the well.
2) Transfer two 7 dpf larvae into each of the wells and fill the wells with egg water (**Fig. 3.3**). Cover the plate with a lid to avoid evaporation and place the multiwell plate in the imaging system 20 min before imaging. The top-lighting will keep the lid free of condensation.

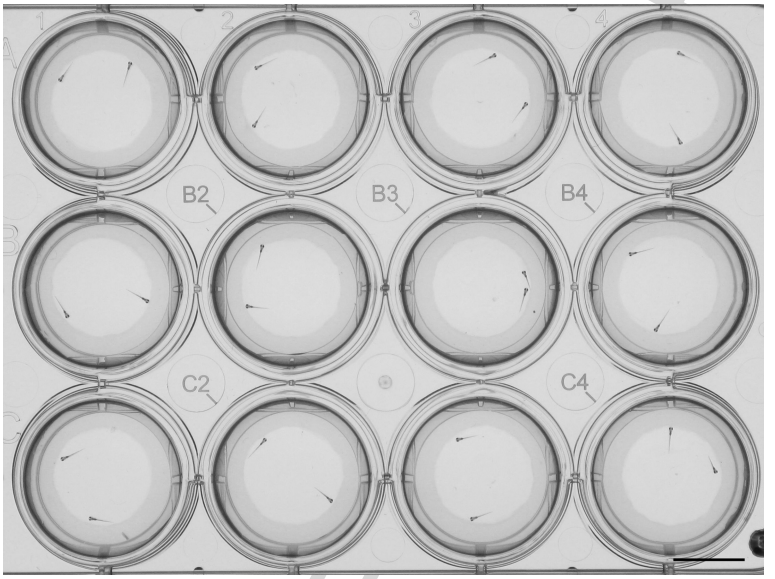

Fig. 3.3. The two-fish assay. The 12-well plate contains agarose rings and two zebrafish larvae per well. The majority of 7-day old larvae are located in different quadrants. Scale bar = 1 cm.

▲ **CRITICAL STEP** It is important to fill the imaging chambers exactly to the rim of the agarose chamber. If a well is filled too low, the images will have a shadow along the edge of the well, which interferes with the automated image analysis. If a well is filled too high, larvae will swim out of the well

.

3) Set the imaging system up in the inverted configuration (**Fig. 3.1C**). Turn the Canon camera on and open "Remote Capture" in the ZoomBrowser software. Select the following settings: maximum optical zoom (10× optical, 1.3× digital), image quality = medium 1 normal (2816×2112 pixels), white balance = fluorescent, iso-speed = 100, aperture (Av) = 6.3, and exposure time (Tv) = 1/30 s.

4) Use the Remote Capture "refresh button" to focus the camera and go to "shooting" to activate the interval shooting mode. Select a 2 min interval and 60 images (2 h recording) to take a sampling of larval positions. Alternatively, select a 6 s interval and 601 images to track larval positions over time (one or two fish per well) or to examine the swimming distance between frames (one fish per well).

5) Acquired images are saved as 0.6 MB JPEGs for further analysis (600 images = 360 MB).

• **TIMING** The two-fish assay takes 1–2 h. The assay may be adapted for multiple 12-well plates for medium- to high-throughput applications, either by imaging two adjacent multiwell plates or by setting up a system with multiple cameras.

3.4. Automated Image Analysis in ImageJ

1) Install ImageJ, either on the imaging computer or on a separate computer dedicated for image analysis. ImageJ is an open source software package that can be downloaded free of charge from http://rsb.info.nih.gov/ij/index.html The Java-based software will work on various platforms. In the Creton laboratory, Image J is loaded on a Dell Optiplex PC (3 GHz, 2 GB RAM) with Windows XP software.
2) Analyze the timelapse recordings in ImageJ as described in **Table 3.1**. Skip step 2 in **Table 3.1** if the images were collected in the upright configuration (bouncing ball assay). The analysis produces a list of coordinates, showing the location and orientation of the larvae. The location of a larva is described by the centroid (X,Y), and the orientation is described by the center of mass (XM, YM), which is shifted away from the dark eyes toward the lighter tail.
3) Measure the midpoint of the dish in ImageJ: (a) open an image in ImageJ; (b) if the image is inverted, flip horizontally/vertically as described in **Table 3.1** step 2, (c) draw a circle matching the edge of the dish (bouncing ball assay)

Table 3.1
Automated image analysis in ImageJ

	Image analysis	ImageJ function
1	Combine 40–60 images in a stack	File, import, image sequence, convert to 8-bit grayscale
2	Flip inverted images (well A1 should be top left)	Image, rotate, flip horizontally/vertically
3	Separate the larvae from the background	Image, adjust, threshold, 0–200 (adjustable[a])
4	Select the dish or a well in the multiwell plate	Tools, circle, drag a circular region of interest
5	Configure the data log	Analyze, set measurements: area, centroid, center of mass, slice number, 3 decimal places
6	Filter out objects that are larger or smaller than the larvae	Analyze, analyze particles, size = 300–600 (adjustable [a])
7	Measure the position of all larvae in all images	Analyze, analyze particles, show outlines, display results
8	Save data logs	Save as: "file name.xls"

[a]Adjust values to include the larvae, while excluding the background.

or agarose wells (two-fish assay); (d) analyze, set measurements, centroid, (e) analyze, measure.

! CAUTION When analyzing large data sets, it is possible to run into memory problems. A stack of 60 JPEG-compressed images requires just 36 MB of storage space. However, the stack of 60 uncompressed images is temporarily stored as a 340 MB file. An additional stack of images is created by the "show outlines" function (**Table 3.1**, step 7), which may be too much for the available RAM in the computer. It is possible to uncheck "show outlines" during the analysis, or to increase the available RAM (see ImageJ, help, documentation, menu commands, edit, memory). Data sets with 600 images may be analyzed in blocks of about 100 images. Alternatively, ImageJ macros can be created to automatically analyze the entire data set, one image at a time (Colwill RM, and Creton R, under review).

3.5. Data Analysis in MS Excel

1) Combine the measurements of the larvae and midpoints of the wells in one MS Excel sheet for further analysis (**Table 3.2**). The X, Y, XM, and YM coordinates are used to determine the location and orientation of the larvae. Apart from the location and orientation, it is possible to calculate the distance between two larvae and the distance between the larvae and the well's midpoint. These measurements can be carried out using the following equations in MS Excel: Distance between larvae $=((X_{L1}-X_{L2})\hat{}2 + (Y_{L1}-Y_{L2})\hat{}2)\hat{}0.5$, Distance between larvae and midpoint (mp) $=((X_{L1}-X_{MP})\hat{}2 + (Y_{L1}-Y_{MP})\hat{}2)\hat{}0.5$. These measurements provide information on the preferred distance between larvae and larval preferences for the center or edge of the well.
2) Use "IF" statements in MS Excel to find the location and orientation of the larvae (**Table 3.3**). The "IF" statements

Table 3.2
Data analysis in MS Excel

	B	C	D	E	F	G	H	I	J	K
49	Copy data from ImageJ								Midpoint of dish	
50	**Object**	**Area**	**X**	**Y**	**XM**	**YM**	**Slice**		**X**	**Y**
51	1	381	1638.986	354.724	1640.506	354.908	1		1484	1074
52	2	346	1984.671	554.272	1985.714	553.227	1		1484	1074
53	3	340	1528.542	640.308	1528.310	641.995	2		1484	1074

The X,Y values in the image range from 0,0 (left, top) to 2815,2111 (right, bottom).

Table 3.3
Calculating the Larvae's location and orientation in MS Excel

Location		
Column M	=IF(D51<J51,"left","right")	Tests if a larva is located in the left half of the dish
Column N	=IF(E51<K51,"up","down")	Tests if a larva is located in the upper half of the dish
Column O	=IF(AND(M51="left",N51="up"),"A","-")	Tests if a larva is located in quadrant A (top left)
Column P	=IF(AND(M51="right",N51="up"),"B","-")	Tests if a larva is located in quadrant B (top right)
Column Q	=IF(AND(M51="left",N51="down"),"C","-")	Tests if a larva is located in quadrant C (bottom left)
Column R	=IF(AND(M51="right",N51="down"),"D","-")	Tests if a larva is located in quadrant D (bottom right)
Orientation of all larvae		
Column T	=D51–F51	X centroid (head) – X center of mass (tail)
Column U	=E51–G51	Y centroid (head) – Y center of mass (tail)
Column V	=IF((T51>0),"right", "left")	Tests if a larvae is facing to the right
Column W	=IF((U51>0),"down", "up")	Tests if a larva is facing down
Column X	=IF(ABS(T51)>ABS(U51), V51, W51)	Main orientation (right, left, up, or down)
Orientation of larvae in quadrant A, B, C, D		
Column Z	=IF(O51="A",X51,"–")	Main orientation in quadrant A
Column AA	=IF(P51="B",X51,"–")	Main orientation in quadrant B
Column AB	=IF(Q51="C",X51,"–")	Main orientation in quadrant C
Column AC	=IF(R51="D",X51,"–")	Main orientation in quadrant D

in Excel include three components: (a) the logical test, (b) value if true, and (c) value if false. For example, the equation **=IF(E51<K51,"up","down")** in column N tests if a larva is located in the upper half of the dish.

3) Use "COUNTIF" statements to count how often a particular location or orientation was observed. For example, the MS Excel function **=COUNTIF(N51:N5000,"up")** will count how many larvae were located in the upper half of the dish during the entire timelapse experiment.
   Count the following parameters in the bouncing ball assay:
   (a) The number of larvae in a specific location (left, right, up, down, A, B, C, D).
   (b) The number of larvae with a specific main orientation (left, right, up, down).
   (c) The number of larvae located in a specific quadrant, with a specific main orientation (e.g., quadrant A, left orientation).
4) Count the following parameters in the two-fish assay:
   (a) The number of larvae located in a specific quadrant, with a specific main orientation (e.g., quadrant A, left orientation).
   (b) The number of larvae that are together in the same quadrant versus a different quadrant.
   (c) The number of larvae with a clockwise orientation (e.g., quadrant A facing up or right)
   (d) The number of larvae with a counter-clockwise orientation (e.g., quadrant B facing up or left).
   (e) The number of larvae at the center versus the edge of the well.
5) Test for significance. Use the Chi-square test to compare the observed versus expected number of larval locations (or orientations). The expected number is based on a random distribution of larvae in the dish. For example, if one acquires 1,200 measurements of larval locations in the bouncing ball assay (30 larvae × 40 images), the expected values are 600 in the upper half of the dish and 600 in the bottom half of the dish. The Chi-square can be entered in MS Excel in the following format: **=CHITEST(observed range, expected range)** The Chi-square test assumes that the measurements are independent. To test if this assumption is correct, carry out the bouncing ball assay with one larva in a culture dish or using longer intervals between frames (e.g., 5 min intervals). If the measurements are independent, the obtained results should be similar to the assay with 30 larvae in the culture dish and a 1 min interval between frames. If the

measurements are dependent, group the measurements of a single bouncing ball assay into one statistical unit. Differences between a control group and an experimental group can then be tested for significance using a two-tailed t-test. For example, carry out the bouncing ball assay in a control group and an experimental group, repeat six times using different clutches of eggs, and calculate the percentage of larvae in the bottom half of the dish. The t-test can be entered in MS Excel in the following format: =**TTEST(array1, array2, tails, type).**

6) When the analysis of the first data set is completed, save a copy of the Excel sheet as a template. This template can be used for subsequent analyses. Copy new data in the template, and MS Excel will automatically calculate the number of larvae with a specific location and orientation.

• **TIMING** The data analysis may take half an hour for a 40-image bouncing ball assay or a day for a 600-image data set of a multiwell plate. The analysis can be sped up by acquiring high-quality images (no shadows, dirt, or bubbles in the background), by collecting smaller datasets (40–100 images), by using ImageJ macros that automatically analyze all wells in a multiwell plate, and by using MS Excel templates that include all calculations, statistics, and graphs.

4. Anticipated Results

In the bouncing ball assay, 6 day old larvae quickly swim away from the bouncing ball. Thus, the number of larvae in the bottom half of the dish should be significantly larger than expected in a random distribution. Larvae in the bottom half of the dish (quadrants C & D) show a preference to face the bouncing ball stimulus (11).

In the two-fish assay, 7 day old larvae prefer to be located in different quadrants and prefer to face outward (e.g., up or right in quadrant B). The percentage of larvae in the same quadrant should be significantly lower than expected in a random distribution. The percentage of larvae with an inward orientation should be significantly lower than expected in a random distribution (11).

The results of these two assays indicate that zebrafish larvae flee from a large, moving shape and avoid close proximity to a conspecific. Avoidance behavior is important for survival because it protects an animal from contact with potentially dangerous or aversive stimuli. A further adaptive advantage is enjoyed by ani-

mals that can anticipate threatening events and social competition through learning and use that information to fine-tune their avoidance strategy. The two assays for avoidance behavior can be modified to examine learning about cues that predict the frequency, timing and direction of a threat (the bouncing ball assay), or the presence and location of a conspecific (two- fish assay). Experiments in the Colwill and Creton laboratories are currently exploring aspects of associative learning in zebrafish larvae using these assays.

Although normal avoidance behavior is motivated by fear of a recognized, specific event, some instances of avoidance behavior may reflect pathologies involving debilitating and chronic levels of anxiety. The developed imaging system can be used to identify larvae with exaggerated reactions to the stimuli used to trigger avoidance as well as larvae with persisting negative after-effects including inactivity, loss of appetite, disrupted sleep cycles and social isolation. Large-scale mutagenesis screens may be carried out in zebrafish to identify genes that control boldness and anxiety. The identification of these genes could provide new insights into the genetics of anxiety disorders in humans. Surveys indicate that nearly 1 in 5 Americans may be affected by anxiety disorders (13) and clinical studies have shown that they are more prevalent among those with a family history of anxiety disorders (14, 15).

In conclusion, the bouncing ball assay and two-fish assay can be used to measure avoidance behavior of zebrafish larvae in response to physical and social stimuli. Medium- to high-throughput screens may be carried out to identify genes, pharmaceuticals, or environmental toxicants that influence various aspects of these responses including latency, duration, frequency, efficacy, and topography.

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Chapter 4

Quantifying Anti-predator Responses to Chemical Alarm Cues

Brian D. Wisenden

Abstract

A behavioral assay of responses to chemical alarm cues can be used to test for cognitive functions related to olfaction, learning, and memory. Alarm cues are chemicals released from damaged epithelial tissue. These cues indicate the presence of predation risk because they are released only after an attack by a predator. Here, I describe a protocol for quantifying behavioral response to these cues and a method for preparing skin extract that contain these cues. In addition, estimates of accuracy and repeatability of these methods are presented for predicting cue potency based on area and mass of skin fillet.

Key words: Behavioral assay, predation, chemical alarm cue, epithelial tissue, stimulus, response.

1. Introduction

The teeth of predators damage the epithelial tissue of their prey. Chemical compounds are released from injured epithelial tissues that are released in no other context. These chemical compounds are known as chemical alarm cues because they reliably cue the presence of predation risk to nearby prey (1–4). Experimenters can use these behavioral responses to test aspects of olfactory function, learning, and memory (5). Here, I describe standard methodology for quantifying these responses and assess and compare two common methods for preparing chemical alarm cue stimulus.

Much of the literature on behavioral chemical ecology of small fishes has been developed using the fathead minnow (Cyprinidae: *Pimephales promelas*) model system (2, 4). The reason for this is that the fathead minnow is native to North America, abundant in the field, and adapts well to lab aquaria. This makes the fathead minnows an attractive study organism

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because results from experimentally controlled laboratory studies can be verified against the ecological realism of field studies. The zebrafish (Cyprinidae: *Brachydanio rerio*) is also in the minnow family, it has similar ecology to the fathead minnow, and thus the chemical ecology literature on fathead minnows applies broadly to zebrafish. The behavioral response to chemical alarm cues by zebrafish has received some attention (6–10). The zebrafish model offers many potential new avenues of research into the genetic and molecular mechanisms of anti-predator responses to predation risk and, indirectly, a means to understand the molecular basis of learning and memory (4, 5, 11). The aim of this protocol is to describe experimental tools, developed largely from the fathead minnow model, to allow exploration of proximate mechanisms of behavioral responses in zebrafish.

Perception of predation risk can be experimentally manipulated by injecting standardized doses of skin extract into aquaria containing test subjects, or in the field by inserting extract-soaked sponges into minnow traps (4). Although many scientists use skin extract experimentally to induce anti-predator behavior, description of stimulus strength has been difficult to standardize because the precise chemistry of the active ingredient(s) is not known. There is evidence that the cue is a ring structure similar to hypoxanthine-3N-oxide (12–14). However, there is not yet consensus that hypoxanthine-3N-oxide, or something similar to it, is the sole active ingredient that induces alarm in these species (15, 16). Indeed, hypoxanthine-3N-oxide has never been confirmed to occur in fish skin. Until these questions are resolved, strength of alarm stimulus is expressed as area (cm^2) of fish skin per unit volume (ml) of solution. When skin is removed from a minnow, the area of the skin fillet is estimated by multiplying the length of the skin fillet by its average width. This method provides a crude estimate of skin area, but accuracy and repeatability of this method has never been quantified. Nor has the method of skin collection ever been described in detail. An alternative method of preparation of alarm cue is to harvest skin, use bibulous paper to draw off excess water, and determine the mass. Stimulus strength expressed as mass (g) per volume of solution (ml) is another increasingly common method of standardizing cue concentration.

2. A Test for Behavioral Response to Chemical Alarm Cues

2.1. Reagents and Equipment

1. Individual zebrafish
2. Sponge filter

3. Airline hosing
4. 60 cc syringes
5. Chemical alarm cue stimulus
6. Test aquaria

2.2. Equipment Setup

Test aquaria. A standard 37-L (10-gallon) all-glass aquarium works well as a test aquarium whereas small test aquaria < 15 L do not because they are too confining. A thin layer of naturally colored gravel improves fish demeanor and reduces stress. The outside walls of the tank should be covered with dark opaque material or painted (we use dark blue in our lab) to prevent fish from seeing into neighboring tanks, which will affect their behavior. Strongly schooling species such as zebrafish copy each other's behavior. Dark walls also provide a sense of security and help test subjects to adjust to being in the test tank. Otherwise, the test tank should contain only a heater and a sponge filter (**Fig. 4.1**). On the front viewing pane, a grid with cell dimensions of 5×5 cm is drawn with a permanent ink pen. These lines are used to score activity and vertical distribution (described below). The dimensions of each cell approximate the body length of adult zebrafish so that the number of lines crossed represents the number of body lengths traveled. A second piece (1.5 m, with internal volume of about 30 mL) of airline tubing is wedged into the rigid plastic lift tube of the sponge filter. This tube is used to surreptitiously

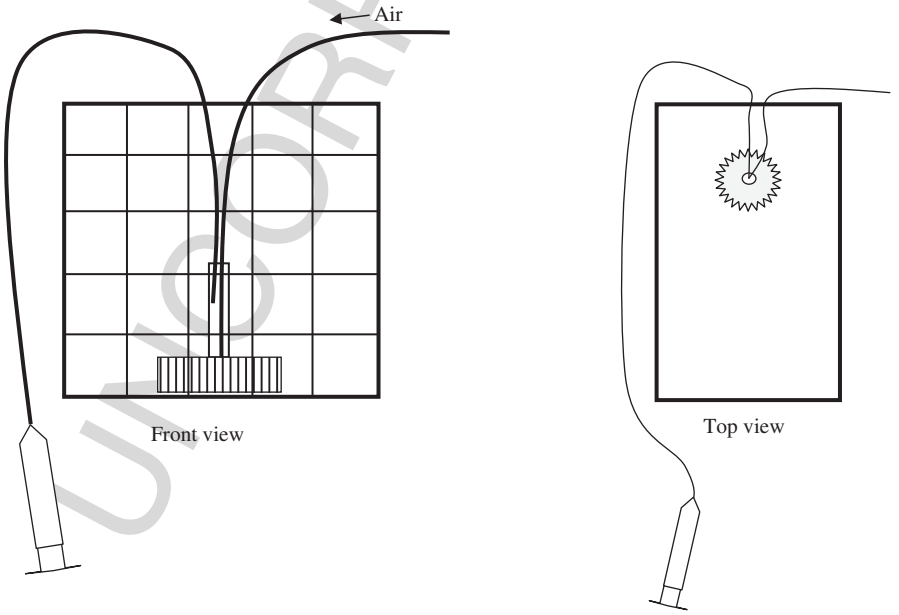

Fig. 4.1. Experimental setup for quantifying behavioral responses to chemical alarm cues.

introduce test stimuli into the test aquarium. Air bubbles and associated water currents issuing from the sponge filter serve to mask stimulus injection and disperse test cue throughout the test tank. Dye tests should confirm that cue dispersal occurs in about 15–20 s.

▲ **CRITICAL STEP** Be sure to kink the syringe-end of the injection tubes and keep them pinched closed using binder clips or other device to prevent these tubes from siphoning the tank dry.

2.3. Procedure

1) Place two test fish in the test tank. Two fish interact and swim throughout the tank. Zebrafish often sulk in the back corner if held singly because in nature they are obligate schoolers, whereas > 2 fish create too much activity to score without the aid of recorded video. Allow the fish at least 24 h to acclimate to the test tank.

   ! CAUTION The behavioral response is based on an increase in the stress state of the test fish, ergo; fish that are already stressed cannot demonstrate a response to test cue. Therefore, it is imperative that test fish are in good health, well fed, and completely acclimated to the test tank. Acclimated fish actively swim about the test tank in a relaxed manner without rapid changes in direction or velocity. Some authors place a black cloth curtain between the observer and the test tank to minimize the influence of observer presence on fish behavior. In my experience, fish are not easily fooled and sense the footsteps of people in the lab even if they cannot see them. Moreover, fish become acclimated to the presence of people in the lab when there is no curtain in place, in which case observer effects disappear.

2) Thaw a dose of pre-prepared chemical alarm cue (see below).

   ! CAUTION Do not microwave or use hot water to speed the thawing process as this may alter the biochemical properties of the alarm cue (17). It is best to either let each aliquot of cue sit on the bench at room temperature, use hand heat to thaw the dose, or place in a small beaker of room-temperature water.

3) Withdraw 60 mL of tank water through the injection tube and discard it. This serves to rinse the tube of stagnant water. Withdraw a second 60 mL of tank water and retain it for later use.

   ! CAUTION Be sure to use fresh stimulus-injection tubing for each trial to prevent residual alarm cue injected in one trial from influencing subsequent trials.

4) Record 5 min of pre-stimulus behavior. The most sensitive behavioral response to risk is reduction in activity. Predators detect prey by detecting motion. To reduce the probability of detection, prey reduce activity (18). *Activity* is recorded as the sum of the number of grid lines crossed by both fish over the 5 min observation period. A simple tally-counter will suffice. More sophisticated apparatus that use the interruption of light beams to record activity have better accuracy with no risk of observer bias. Prey in the open water column are conspicuous to predators while those that seek refuge on the bottom are less so. Therefore, another common anti-predator behavioral response is to move to a lower position in the water column (19). Each row in the grid is assigned a value: "1" for the top row on down to "5" for the bottom row. *Vertical distribution* is recorded as point samples taken every 15 s of the row occupied by each fish.

5) Inject test stimuli by attaching a syringe containing test cue to the injection tube. Slowly and gently depress the plunger of the syringe to force the cue into the injection tube.

   ▲ **CRITICAL STEP** Be sure to depress the plunger gently, especially at the beginning and end of the injection. High-velocity injections can induce a fright reaction because fish respond to abrupt changes in water pressure.

6) Fold over the end of the injection tube to prevent back flow of test cue, detach the cue syringe from the injection tube, and attach the syringe containing the previously retained 60 mL of blank tank water. Unfold the injection tube and gently flush the test cue from the injection hose and into the test tank. The entire injection process should require about 60–90 s to complete.

7) Immediately record another 5 min of activity and vertical distribution behavior as before.

2.4. Anticipated Results

Calculate change in behavior by subtracting post-stimulus behavior from pre-stimulus behavior. This method of analysis uses each tank as its own control and removes inter-tank variation (which can be substantial) from analysis of response. Non-parametric analyses (e.g., Mann-Whitney U test, Kruskal-Wallis one-way ANOVA) are recommended because these behavioral data typically do not meet the assumption of normality required by parametric statistical procedures. Sample data (9) from zebrafish are presented in **Fig. 4.2**.

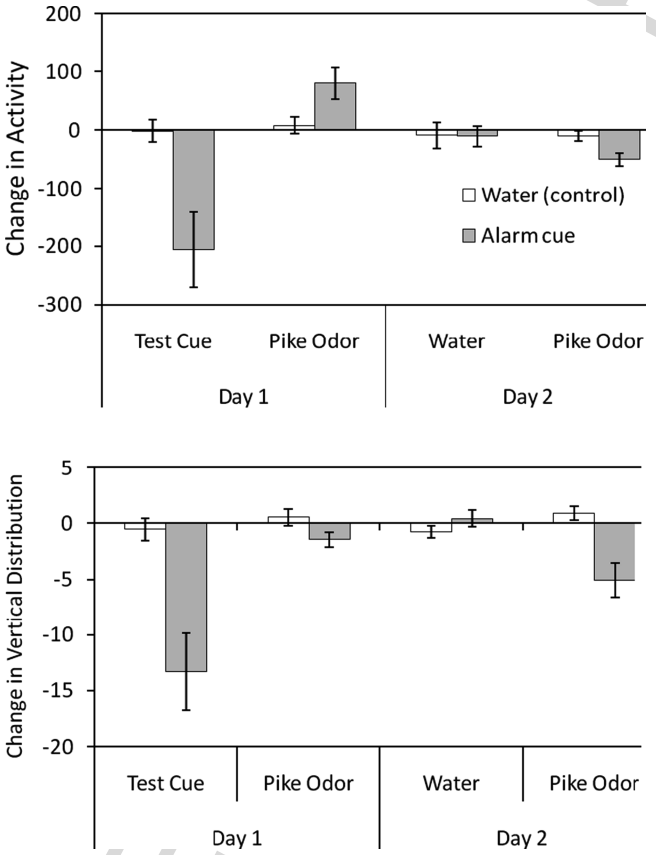

Fig. 4.2. On Day 1 zebrafish were presented with one of two test cues: either water (control) or skin extract. After 5 min they were given pike odor. Zebrafish responded with a reduction in activity (**a**) and movement to the bottom (**b**). The water in test tanks was replaced with fresh water and the same fish were retested on Day 2. Fish gave no response to water on Day 2, but those that had been allowed to associate skin extract with pike odor the previous day significantly reduced activity (**a**) and position in the water column (**b**), indicating learned recognition of pike odor. Data from Korpi and Wisenden (9).

3. Preparation of Chemical Alarm Cues

3.1. Reagents

1. Donor fish
2. Glass Petri dish 15 cm in diameter
3. Single-bladed razors
4. Two pairs of fine-pointed forceps
5. Ruler, paper towel

6. Beaker on bed of ice
7. Blender or tissue homogenizer

3.2. Description of Stimulus Preparation

The best surgical platform is a smooth wet surface, such as a wet overturned large-diameter glass Petri dish, so that skin does not abrade during filleting. Place a live fish on the glass surface and carefully but firmly hold it against the surgical platform. Kill the fish with a single chop with a one-sided razor blade or scalpel through the epaxial musculature and spinal column, effectively decapitating it. On IACUC forms this is called cervical dislocation. It is helpful to leave the head attached to the rest of the body by the hypaxial musculature, because a headless minnow is difficult to hold during the later cuts. Filleting a minnow follows the same principles as filleting a large game fish. Because minnows are small fish, well-developed fine motor skills, or good "lab hands" are needed to efficiently remove skin intact and in a timely manner.

Because odor discrimination among different test cues is often the focus of study, anesthetic is not used because it could potentially confound the chemical signature of skin extract. The total length of the fish is measured after it has been killed, but before skin has been removed.

Holding the head between left thumb and forefinger, a superficial cut is made with a one-sided razor blade along the dorsal surface starting at the anterior end, along the left margin of the dorsal fin and ending at the dorsal surface of the caudal peduncle. Then, a cut ventrally and diagonally from the anterior end is made to circumscribe the pectoral girdle, continuing posteriorly along the dorsal margin of the pelvic girdle and ending on the ventral edge of the caudal peduncle. One pair of fine-tipped forceps is used to anchor the epaxial musculature while a second pair of forceps is used to grasp the anterior edge of the cut epidermis. A series of gentle posteriorly directed tugs, pausing every 5 mm or so to reposition the second pair of forceps at the point where the skin separates from the musculature, is used to peel the epidermis from the underlying muscular in a single sheet. With practice, the skin peels off quickly and easily. At the caudal peduncle the skin fillet is torn free from the base of the caudal fin. To measure the area of the fillet, the skin fillet is spread out on the wet surface of the glass dish until it resembles its original shape. A plastic ruler is used to measure the maximum length of the fillet and the width of the fillet at an intermediate point of approximately average width. The product of length and average width is the estimated area of each fillet. Wet weights are measured by briefly placing the fillet on moist paper toweling to draw off excess water and then weighing the skin to the nearest milligram. The skin fillet is then placed in a beaker of dechlorinated water resting on a bed of crushed

ice. Chilling the solution prevents biochemical decomposition of alarm cue (17).

Removing the skin fillet from the second side of the fish is more difficult because the head usually tears completely free of the body during the removal of the first fillet. The most time-efficient and effective method for removing the second skin fillet is to use forceps directly without making any new cuts. The epaxial musculature is grasped with one set of forceps, while a second set of forceps is inserted as near to the anterio-dorsal edge of the skin to peel the skin free from the dorsal fin. Once free of the dorsal fin, the skin separates easily from the underlying musculature and tears free from the body at the base of the caudal peduncle. The pelvic girdle often remains attached to the skin fillet and is removed with the razor after the skin fillet is removed from the fish. Any viscera that remain attached to the skin are easily pulled free with forceps. The second skin fillet is spread out on the smooth wet glass, measured and weighed and then placed in the beaker of chilled dechlorinated water.

Although 1 cm^2 of fathead minnow can be diluted to 58,000 L and still evoke overt anti-predator behavior (19), the typical dose for a 37 L aquarium is 1 cm^2. Therefore, plan in advance of stimulus collection the number of treatments and replications needed for each experiment and then harvest skin extract until enough has been collected to make doses of 1 cm^2 per trial. Because behavioral responses to alarm cues are highly variable, we generally conduct 15 trials per treatment to ensure sufficient statistical power to detect an effect.

Blend the skin extract using a tissue homogenizer (an inexpensive kitchen blender works just as well) and then filter out the scales and connective tissue. Avoid using filter paper because the pores will clog with scales and mucus. A loose wad of polyester fiber will suffice, or a quick spin in a large volume centrifuge tube will pull tissue fragments out of solution. Dilute the filtrate (or supernatant, depending on method of filtration) to the final volume desired. For a 37-L test aquarium, dose volume is typically 10 mL. Aliquot test cue into 10-mL doses and freeze at –20°C. The cue is thawed one aliquot at a time as needed. Making alarm cue as a large batch ensures consistent cue concentration for all trials within an experiment.

4. Reliability of Area Estimates and Wet Mass

These data were collected from fathead minnows, *Pimephales promelas*. However, results from fathead and zebrafish are both obligate schoolers and use chemical information about risk in

similar ways. Results derived from fathead minnows apply equally to zebrafish.

Fathead minnows were purchased from a local supplier of bait fish and transferred to a 555-L holding tank with two biological filtration towers and recirculating flow rates of 500 L/h each. Fish were fed standard commercial flake food and maintained at 20°C. Skin extract was prepared on two separate occasions using 40 minnows each time. All methods reported here were approved by Minnesota State University Moorhead IACUC protocol # 02-T-BIOL-015-N-R-1.

Skin fillets were measured in area and mass and then placed in a dry microcentrifuge tube, capped and kept on ice. Each fish produced two fillets and the sum of the areas generated an estimate of skin area for each fish. To determine dry weight, microcentrifuge tubes were placed in a centrifuge (Heto VR-1 Mini St. a.) at room temperature (*ca*. 20°C) and spun at high speed for 7 h. The centrifuge was attached to a vacuum pump (Alcatel Pascal 2005 SD) with a moisture trap (Heto CT 60E). After 7 h the resulting pellets were weighed to the nearest milligram.

Fish were 59.4 ± 0.67 mm in length and each fish produced an average of 489 ± 21 mm^2 of skin. Each fish produced an average of 231.7 ± 10.3 mg of wet skin and 39.0 ± 2.0 mg of dry skin. Total length (**Fig. 4.3**) predicted 55% of the variation in fillet area and 44% and 41% of fillet mass (wet and dry, respectively). Fillet area (**Fig. 4.4**) was a better predictor of wet and dry weight

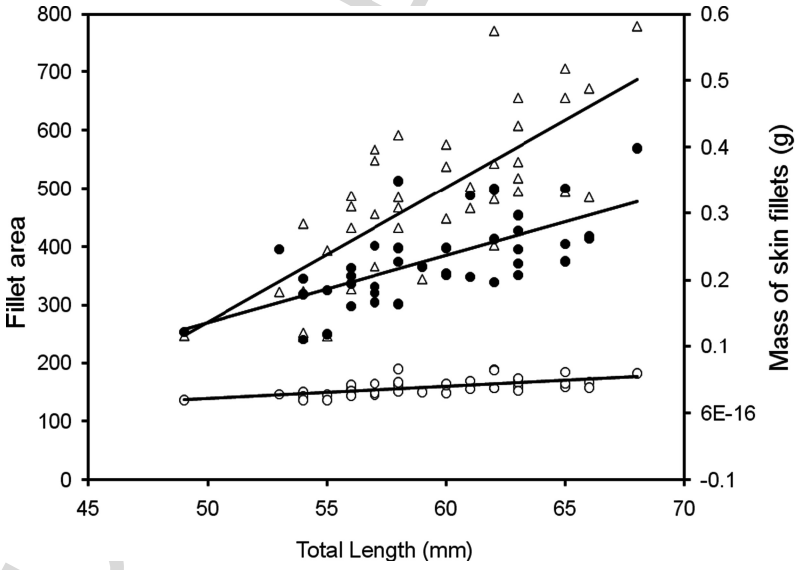

Fig. 4.3. The area of skin (mm^2) per fish (sum of *left* and *right* sides) and the corresponding wet and dry weights (g) of skin as a function of total length (mm). Regression lines are TL = 23.112 (area) – 884.9, $R^2 = 0.559$, $F_{1,39} = 48.13$, $p < 0.001$; TL = 10.120 (wet wt) – 369.7, $R^2 = 0.436$, $F_{1,39} = 29.14$, $p < 0.001$; TL = 1.862 (dry wt) – 71.7, $R^2 = 0.407$, $F_{1,39} = 26.05$, $p < 0.001$.

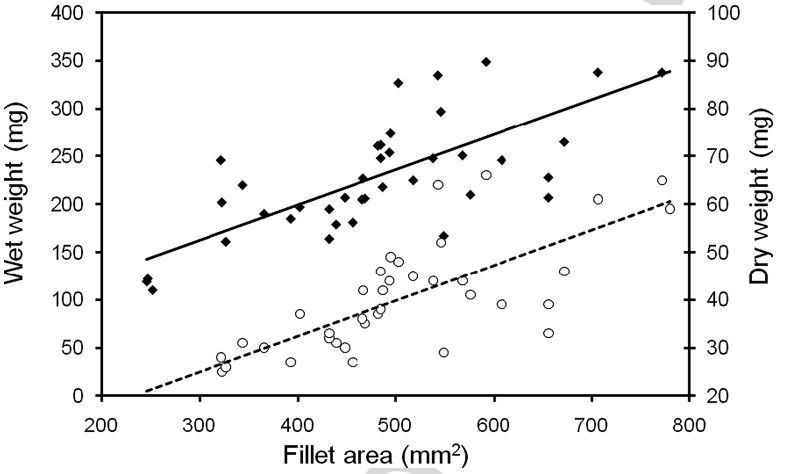

Fig. 4.4. Wet and dry weight (g) of skin fillets per fish (sum of *left* and *right* sides) as a function of estimated area (mm^2) of the skin fillets. Regressions: Area = 0.369 (wet wt) + 51.6, $R^2 = 0.553$, $F_{1,39} = 47.10$, $p < 0.001$; Area = 0.071 (dry wt) + 2.8, $R^2 = 0.616$, $F_{1,39} = 60.84$, $p < 0.001$.

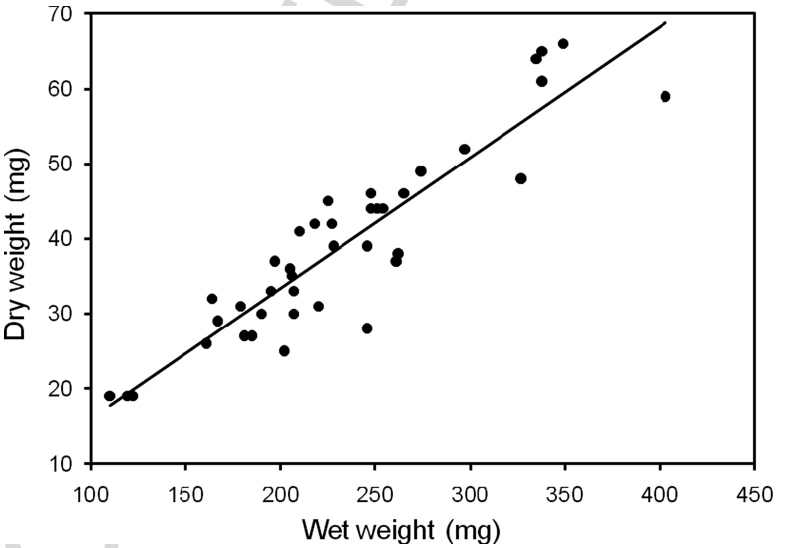

Fig. 4.5. Dry weight (g) of skin fillets per fish (*left* + *right* side) versus wet weight (g) of skin fillets per fish (*left* + *right* side). Regression: Wet wt = 0.174 (dry wt)–1.5, $R^2 = 0.838$, $F_{1,39} = 196.80$, $p < 0.001$.

of skin than simple total length; however, variation in skin area still only predicted 55.3% and 61.6% of the variation in wet and dry weight of the skin, respectively. Wet weight was a better, but not perfect, predictor of dry weight with an accuracy estimate of 83.8% (**Fig. 4.5**).

In my hands, ability to predict stimulus strength (dry mass) from skin area was only 56% – a rather low degree of accuracy and repeatability. The ability to predict dry weight of skin from its wet weight was 84%. For the modest additional effort required

to collect wet weight, an obvious recommendation for workers in this field would be to describe stimulus strength on the basis of wet weight of skin per volume of solution. Error in the relationship between fillet area and dry weight comes in part from the difficulty in estimating fillet area. Fillets are irregular in shape and visual estimation of the average width was likely the source of most of the error. Additional error may have occurred from muscle tissue adhering to the skin, especially at anterior end of the fish. Muscle tissue inflates the ratio of wet and dry weight relative to fillet area. Variation in the degree to which excess water was removed before measuring wet weight may have been responsible for error in predicting dry weight from wet weight.

Another method for collecting chemical alarm cues is by lightly scoring the flanks of a freshly killed fish with a scalpel or razor blade and then rinsing with a standardized volume of dechlorinated water. This method simulates the scraping action of predator teeth across the skin of prey. This method is used in field studies where cue is prepared on site for each trial and used immediately (20). Although the number of cuts per flank can be standardized, the amount of epidermal tissue affected and the amount of alarm cue released is much more difficult to quantify than by the method of estimating area or mass of skin fillets. Until the chemically active components in minnow alarm cue are characterized with certainty and a method devised to measure them, the best way to describe alarm stimulus strength is to use wet weight of skin fillet per volume of water.

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Chapter 5

Modified Associative Learning T-Maze Test for Zebrafish (*Danio rerio*) and Other Small Teleost Fish

Georgianna G. Gould

Abstract

Associative learning is a form of classical (Pavlovian) conditioning in which a neutral stimulus (e.g., the color green) is paired with a stimulus of some significance to an animal (e.g., food), such that for the animal, the color becomes synonymous with food to evoke the same innate, reflexive behavioral responses (e.g., food seeking). This protocol is designed to test the acquisition and extinction of reward-visual stimulus association in zebrafish (*Danio rerio*) in a T-maze. It is based on the visual discrimination protocol of Colwill et al. (2005), in which colors or patterns are paired with a food reward. The protocol has been modified to include a reward box within the T-maze aquarium so that the influence of drug reinforcement can be studied without the potential confound of reward residues contaminating the testing arena.

Key words: Classical conditioning, stimulus, response, acquisition, extinction, T-maze test.

1. Introduction

Associative learning and addiction to psychomotor stimulants are complex behaviors involving the mesolimbic pathway (1, 2). Elevated extracellular levels of dopamine, serotonin, and their interactions can modulate the tone of central reward circuits (3–5). Dysfunction of dopaminergic and/or serotonergic systems can impair associative learning task performance and may underlie anhedonia, the inability to experience pleasure, a symptom frequently associated with depression, schizophrenia, and other psychiatric disorders (6, 7). Consistent with psychiatric translational research goals, if drug actions can be examined in animal models

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exhibiting behavioral parallels to core disorder symptoms, their clinical effectiveness might be better predicted and understood.

Chronic unpredictable stress (CUS) or developmental exposure to organophosphates can produce behavior consistent with anhedonia in rats, such as reduced consumption of sweet solutions (8, 9). Sustained antipsychotic (e.g., olanzapine) or serotonin reuptake inhibitor (e.g., fluoxetine) administration to rats appears to prevent or reverse CUS-induced anhedonia (7, 10). However, more efficient and cost-effective animal models are needed to screen novel treatments and identify genetic or environmental factors contributing to psychiatric disorders, addiction, or impaired learning during critical developmental stages (6, 7). In order to screen for genes influencing reward-seeking behavior and addiction, targeted genetic manipulations of biogenic amine system components can be performed in zebrafish (11). Since zebrafish produce thousands of eggs that develop into adults within 3–4 months, they are amenable to large-scale use in behavioral tests following genetic or pharmacological/toxicological manipulations (11, 12). Zebrafish pharmacological studies often parallel results from rodent behavioral tests, and also implicate dopamine and serotonin system involvement in associative learning and addiction (12–15).

Zebrafish can perform basic learning and memory tasks, and reward-seeking neural pathways have been described in them. While mesolimbic circuits are not structurally conserved among teleosts and mammals, the lateral and medial telencephalic pallium appear to function in a homologous manner to the hippocampus and other components of mammalian mesolimbic pathways (16–18, 21). Further, teleost and mammalian biogenic amine regulatory systems function similarly; most neurotransmitter receptors and transporters share similar binding site properties and responses to pharmaceuticals (19). Associative learning tests for food reward have been performed in zebrafish by several lab groups (16, 20–22), all of which have demonstrated that with 10–25 association trials carried out over days or weeks, zebrafish can acquire and retain color, light, and spatial cue reward associations.

Presented herein are slight procedural modifications to a zebrafish T-maze visual discrimination learning-extinction-reversal task (20) that expand its utility from food-reward to psychostimulant-reward strength and association studies. The zebrafish visual discrimination test is an associative learning task employing Pavlovian (operant) conditioning in which food or a psychostimulant reward is paired with a colored background in successive learning trials until a conditioned association is formed between the reward and a color in the maze (20). The modified protocol utilizes the offset cross maze (Ezra Scientific, San Antonio, TX) in a T-maze configuration in which the two T-side arms off the top are lined with different colored sleeves,

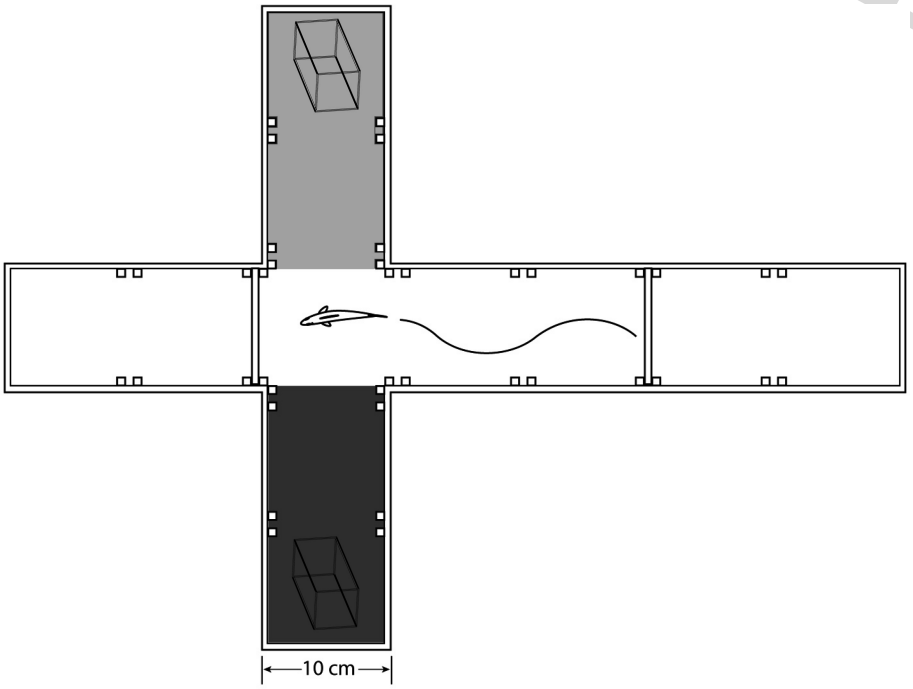

Fig. 5.1. Color-reward association T-maze configuration of the offset cross maze. The top 20 cm long section off of the center is sealed off with a drop in door, and *left* and *right* arms are lined with either *green* (lighter shade of grey) or *purple* (darker shade of grey) poly sections. After each trial, the colored arm liner location is randomly alternated. Hence if left is lined with green and right with purple in trial 1, this pattern may be reversed for trial 2. Colored plastic reward boxes (AMAC, The Container Store, USA) with tone matching the poly liners in the arms are placed at the ends of each arm, with open ends facing outward.

with matching colored plastic boxes inside, only one of which is paired with a reward if the fish swims into it (**Fig. 5.1**). Rate of acquisition of the association can vary under different conditions, and its strength can be measured by running successive trials without rewards until the reward-seeking response is extinguished (20). This modified associative learning test for zebrafish may be particularly useful for studying the effects of genetic manipulation, drug or toxin exposure on cognition.

The modification is the addition of removable 5.9×5.9×10.6 cm colored plastic reward boxes (AMAC, The Container Store, USA), which can be removed from the maze to administer dissolved alkaloid substances such as nicotine, caffeine, morphine, or cocaine. Administration of drug reward to fish in the boxes allows better control of exposure length and reduces contamination of the maze water with dissolved drug rewards. However, this modification necessitates additional pre-training of the zebrafish to swim into plastic boxes for a food or drug reward. Other fish species, such as goldfish (*Carassius auratus*) or fathead minnows (*Pimephales promelas*), can also perform this task, and such studies could aid evolutionary and environmental biologists as well as basic and translational behavioral neuroscientists.

2. Materials

1. Several 500 mL–1 L beakers filled with habitat water (two per treatment group, for acute drug pre-exposure and rinsing fish after drug reward treatment)
2. Offset cross maze (Ezra Scientific, San Antonio TX, www.EzraScientific.com)
3. Conditioned habitat water at ≈ 25°(or close to the temperature of the home tank)
4. Electric aquarium heater [submersible stick style (e.g., Theo, Hydor, Italy)) to maintain maze water temperature if necessary (optional)]
5. Adult zebrafish for testing (sample sizes in the range of 8–12 are recommended)
6. Drug or test compound of interest
7. Two digital timers (one to track total run time, the other to track each trial)
8. Digital camera (s), (software such as Stoelting Anymaze™ or Ethovision® by Noldus can also be used)
9. Index cards for hand scoring or computer with spreadsheet opened to collect data
10. Purple and green, or blue and red matte polypropylene folders of moderate brightness, cut into liners to fit inside the arms of maze (six 10×10 cm and two 10×20 cm liner sheets of each color) (Office Max, USA).
11. Medium-sized binder clips, 12
12. Copy stand or camera tripod to mount digital camera above maze
13. Dip nets, 1–2 (additional nets required for each drug exposure)
14. Purple and green, or blue and red, plus clear 5.9×5.9×10.6 cm plastic boxes with lids off (AMAC # 10022870 for purple, 10022872 for green, 10022869 for red, 10022873 for blue, 60380 for clear from the Container Store, USA) www.containerstore.com)
15. Frozen bloodworms, baby brine shrimp or flake food for food reward
16. Caffeine, morphine, cocaine, nicotine, or other addictive stimulant for drug reward
17. Notebook or spreadsheet for data (record outcomes and trial times)

3. Experimental Timeline and Suggested Time Allowances

Depending on the goals of the research, experimenters may opt to perform the entire acquisition-extinction-reversal task, examine acquisition and extinction only, or just test acquisition. The following experimental timeline includes all learning task components that were performed in the initial design of the test (20), plus the additional conditioning steps required to train fish to enter the reward administration boxes.

3.1. Timing and Timeline for Zebrafish Associative Learning in T-Maze

Step 0 – Food restriction for 24–48 h prior to training – Day-1 (omit if drug is reward)

3.2. Pre-training (4 Days): Days 0–4

Step 1 – Conditioning in home tank containing clear plastic box (2 days) Days 0–2 Observe a group of six fish in their home tank (for 40–60 min on average) and remove the reward box and give reward to all fish that swim into the clear plastic box, stop the training session when all fish in the home tank have been administered at least 3 rewards.

Step 2 – Conditioning in T-maze with clear boxes, no arm liners (2 days) Days 2–4

3.3. Discrimination Task Acquisition (16 Days, 1 Session/Fish/Day): Days 5–21

Step 3 – Training in T-maze in which side arms are lined with purple and green poly sheets and coordinated colored boxes are placed at the end of each arm. Each day train fish through 4 trials of 10 min each to swim to their designated color arm and enter the plastic box to get a food or drug reward. For each trial the correct color for reward association is altered in a random pattern between the right and the left arms (in a L, R, R, L, R, L, L, R pattern (20)), while the fish is confined in the start box. Incorrect color choices are followed by a 10 min correction round in which the wrong colored arm is closed off by a drop-in door, so the only option for the fish is to swim into the "correct" colored box and get a reward. The next trial immediately follows the correction round, and the correction round itself does not constitute a trial round. A training session for one fish can take as long as 80 min to perform, but on average early training sessions take 40 min and later sessions take 25 min per fish to complete. House fish trained to each color separately.

3.4. Extinction of Association (Variable Timing, Roughly 10 Days) Days 22–32, May Vary

Step 4 – In T-maze in which side arms are lined with purple and green and colored boxes are present at the end of each arm, but no rewards are given and no correction trials are performed for incorrect choices. Behavior of each fish observed in 4 trials per day, over as many days as are required for fish to swim into

the incorrect box 50% of the time. Average time per session will initially be ≈20 min and will later approach 40 min.

3.5. Reversal of Discrimination Learning (16 Days, 1 Session/Fish/Day) Days 32–48

Step 5 – Performed as per discrimination acquisition, but "correct" color is switched (20).

4. Equipment Setup

The offset cross maze, available from Ezra Scientific (San Antonio, TX, www.EzraScientific.com) is configured for use as a T-maze for the color-reward associative learning task by closing off the top short arm (**Fig. 5.1**). For the associative learning task, the 10 cm^2 end section of the long arm serves as the starting box for the fish, and the two opposing 20 cm^2 T-side arm sections opened. For acquisition, one T-side arm is lined with purple poly folder sections on the three inner sides and bottom and the other arm is lined with green sections. The maze is 10 cm deep, and should be filled to a uniform depth of 5 cm with 3.5 l of home tank water. Water temperature should be maintained within 2°C of that in the home aquarium (we use ≈25°C), and can be heated, if necessary, by adjusting the room temperature or by using a submersible heater (Theo, Hydor, Italy) fixed to the bottom of the T-maze runway (20). Poly sections are fastened to the T-maze side-arm wall with binder clips and submerged and pressed onto the maze bottom. Colored plastic reward boxes (AMAC) should be added to their matching colored arms. The maze can be either placed on a copy stand (Kaiser RS1, B&H Photo, New York, NY) with a digital camera (we use an HP Photosmart R742) mounted to it, or the maze can be placed on the floor with a digital camera mounted above it on a tripod. Behavioral testing is generally carried out between 0800 and 1,700 h under fluorescent light, and our fish are housed on a 14:10 light dark cycle with lights on at 0700 h CST.

5. Optional Acute Drug Pre-exposures

Depending on the research question, it may be of interest to study the effects of pre-exposure to drugs that either block or enhance mammalian reward-seeking behavior. Such exposures might be performed acutely through a bath exposure repeated each day prior to acquisition training and/or extinction testing.

We house our zebrafish in groups of six in 3 L tanks of a benchtop aquatic habitat (Aquatic Eco-Systems, Apopka, FL) with recirculating filtered deionized tap water, 27°C, supplemented with 200 mg/L Instant Ocean® synthetic sea salts (Spectrum, Atlanta, GA). Zebrafish can be pre-exposed to drug(s) dissolved in 250–300 mL of habitat water from this system in a 600 mL beaker each day prior to training and task acquisition. Acute bath exposure duration to water-soluble drugs or chemicals is typically 3–5 min. In studies examining the effects of acute bath exposure of zebrafish to anxiolytics, this exposure duration was adequate for the compounds to reach target sites in the brain and alter behavior (23, 24) (*see* also Chapter 8). Since fish must complete 4 trials of up to 10 min each, it is likely that maximal drug effect will occur during the second trial and could diminish by the fourth (24). Solvents such as DMSO, acetone, or ethanol can be used, preferably at the lowest concentration necessary. If a solvent is used, it is important to run a vehicle control group of animals since such solvents will increase mobility, arm entries, and may either impair or enhance task performance. After acute exposure, transfer the zebrafish to a holding beaker for 5 min to allow the drugs to reach their targets and take effect prior to testing.

6. Procedure

(1) Food restriction: If examining the association response to a food reward, cease feeding zebrafish for 24–48 h prior to pre-training. Skip this step if you are using a drug as a reward, it may not be necessary and could confound results.

(2) Pre-training:

a. Step 1 – Use a clear plastic (AMAC) box in each home tank. Place opened plastic box in bottom of the 3 L housing tank with six zebrafish of mixed gender. Observe for 40–60 min. When any fish swim into the box, quickly remove the box and administer reward (add food or pipette in an appropriate dose of drug reward). Stop the pre-training session when all fish have been rewarded 3 times. Repeat this step 24 h later.

b. Step 2 – Use clear plastic boxes placed in the ends of the unlined T-maze arms. Place the T-maze on a white or light-colored countertop, floor, or surface, and fill with 3.5 l water. CAUTION! Check the maze water temperature, adjust if >2°C cooler than habitat water temperature by making the room warmer or using a submersible aquarium heater.

i. Add clear plastic boxes to either end of the T side arms and place zebrafish in the start box (box at far side of extreme long end) for 5 min. Lift the drop in door to the start box, watch for the fish to exit, and gently drop the door down after the fish leaves the box and start the timer.

ii. Observe as the fish swims through the maze until it enters a clear plastic box in either one of the T-maze side arms.

iii. When the fish enters a box, stop the timer, gently tilt the box upright, and remove the box with fish from the T-maze to administer the reward. Record the elapsed time.

iv. After reward is administered (either food has been eaten or 3–5 min of drug exposure), gently dump the fish and reward-containing water into a dip net. Place the dip net in a beaker of clean habitat water to dilute or remove any residual drug or food reward from the fish and net.

v. Return the fish to the start box. Seal off the side T-arm with the reward box that was previously selected by the fish, so that only the clear box on the other side is available. Open the start box drop-in door and start the timer. Stop the timer when the fish enters the opened top arm, tilt the box upright, remove it from the maze, and administer the reward.

vi. Repeat steps iii.–iv. through four trials per fish per day.

vii. If training multiple fish, change the habitat water between fish, but for any one fish use the same water for all four successive trials.

viii. Repeat the Step 2 task (i–vi) at the same time on the following day.

(3) Discrimination Task Acquisition (color = food reward):

a. If pre-exposing zebrafish to drugs or compounds, prepare a bath solution of the compound (dissolve drugs in 250–300 mL of habitat water) in a beaker. Prepare a second bath for untreated controls of just habitat water. If a solvent such as DMSO is necessary to dissolve the compound, then a vehicle control exposure should be prepared in addition to the habitat water control. Add fish to the beaker and expose for 3–5 min. Maintain the same exposure time for all fish. Following exposure, place the fish in beaker of drug-free habitat water for an additional 5 min for the compound to take effect. NOTE: Skip this

step and proceed to step 2 if you do not intend to test a drug pre-treatment.

b. Set up your camera, if you are video-recording or using video-tracking software so that the entire T-maze is in view.

c. Divide each treatment group of fish such that 50% are trained to swim to the purple arm and box for a reward, and the other 50% are trained to swim to the green arm and box for a reward. Keep these groups distinct when housing the fish in home aquaria.

d. Set up the T-maze with colored arm liners and reward boxes as in "EQUIPMENT SETUP". Fill with 3.5 L of habitat water. Place maze on white or light-colored surface. Close the drop in door of the start box. CAUTION! Press and smooth the poly arm liners onto the maze bottom to remove air bubbles so that they do not float up. Also make sure there are no gaps between the poly squares and the sides of the maze that zebrafish might swim behind.

e. Place the fish in the start box and allow it to acclimate for 5 min.

f. Start the camera if recording. Open the drop-in door of the start box and watch for the fish to swim out. Gently replace the door to prevent the fish from re-entering the start box, and start the timer. Observe as the fish swims in the runway, until it enters one of the T-maze side arms, and a reward box. Stop the timer when the fish enters a reward box, or after 10 min, the end of the trial.

   i. If the fish enters the "correct" colored arm and reward box, flip the box up and gently remove it from the maze. Administer the food or drug reward to the fish. After the reward has been consumed or taken up for 3–5 min, gently pour the water containing the fish into a dip net over a sink (or waste collection bottle if the substance is hazardous) to drain. Place the fish in the dip net into a clean beaker of habitat water to wash. This constitutes a successful trial.

   ii. If the fish enters the "incorrect" colored arm and reward box, flip the box up and gently pour the fish back into the start box. Seal off the "incorrect" colored arm with a drop in door, for a correction run. Open the door to the start box and start the timer. When the fish enters the "correct" colored arm, reward it promptly as in step (i.) above. This constitutes an "incorrect" trial. Note: The time for

this trial will include the time prior to the incorrect box entry PLUS the time to complete the "correction" run (e.g., 125 s before wrong box entry, plus 300 s to enter correct box, yielding 425 s for one trial with an "incorrect" outcome). However, both times should be recorded separately, so that "time to mistake" and "time for correction" can be analyzed separately.

iii. If the fish does not enter either arm after 10 min, remove the fish from the maze and replace in the start box. This constitutes a failed trial. CAUTION! Some fish may not perform this task well for a number of reasons, including being satiated if the reward is food, injuries or mutations affecting vision, locomotion, or their ability to eat. Observe the fish in its home tank to determine if it appears impaired and should be eliminated from the subject pool. Some healthy fish will fail on initial trials, and perform the task in later trials or on subsequent days. If such a fish fails to perform the task after 2 days of trial, it might be prudent to substitute it with another fish, and make note of it when analyzing the data.

g. Repeat step e. three more times, frequently switching the correct color in the T-side arms between left and right for the fish. The pattern L, R, R, L, R, L, L, R was suggested in the original design (20). After each trial make note of the times to complete each trial and outcomes of the four trials for each fish for each training day. The possible data outcomes for % correct will thus be 0, 25, 50, 75, or 100. The time should be recorded in seconds and will fall between 10 and 600 s for each trial.

h. After completing 16 sessions of acquisition training for all fish in the study groups, calculate for each day the mean and standard error for % correct and time to complete runs. This data may be analyzed by repeated measures ANOVA. For post-hoc analysis of significant results either Fishers LSD, Tukey's HSD, Scheffe's test, or equivalent may be used. However, as the % correct data are discrete, Mann-Whitney U-tests are even more appropriate to use for post-hoc analysis.

(4) Extinction of the Acquired Association:

a. The goal of this component of the procedure is to acquire a measure of the strength of the association. Set up and perform the experiment as for "3. Discrimination Task Acquisition", except do not reward or correct fish for any outcome. Trials remain 10 min long; but

when fish complete the task, either "correctly" or "incorrectly" by swimming into a box, stop the timer and then return the fish to the start box. Switch the colors in the arms of the T-maze and proceed to run the next trial, without rewarding or correcting the fish. Complete 4 trials/session/day.

b. The duration of days of extinction testing is variable. When the fish no longer displays a preference for the formerly "correct" color choice that is greater than 50% over three successive sessions, the association is lost. Extinction testing may cease at that time and data is analyzed as for "#. Discrimination Task Acquisition". Previously fish trained to purple for a reward retained the association through 7 extinction sessions, while fish trained to green lost the association in that time (20).

c. Alternative approaches to evaluating extinction might include waiting for several days or a week between acquisition of the association and the extinction tests.

(5) Reversal of Discrimination of Color Association

a. The goal of this component of the procedure is to retrain the fish that were previously conditioned to associate, for example, purple with a food or drug reward to now associate green with that reward. Purple, in this same example, is then treated as "incorrect" and green as "correct".

b. With the exception of the reversal of color cuing reward, the procedure is carried out exactly as in "3. Discrimination Task Acquisition". The number of training sessions required for reversal of the association may be less than 16. In previous studies the fish were more efficient at acquiring the new association (20). Discretion should be used in determining when to end the training trials and proceed to extinction tests.

c. Depending on the research goals, it may be preferable to opt to perform discrimination task acquisition and immediately follow it with reversal of the discrimination task, if plasticity in learning is of interest.

(6) Variations on Association Cues

a. As previously demonstrated, other color pairings such as red and blue may be used instead of green and purple (20). Zebrafish have four distinct cone photoreceptors with maximal sensitivities to light wavelengths of 360, 417, 480, and 570 nm, so other color combinations at these wavelengths may also work for discrimination learning (25). Zebrafish can distinguish between red and blue to avoid aversive stimuli, and other tests have revealed behavioral sensitivity peaks at 520, 360,

420, and 600 nm (26, 27). Untested color and pattern combinations should be studied in pilot tests similar to the studies performed to assess blue and red perception (25) before proceeding with the lengthy acquisition of association procedure.

b. Patterns of black and white or pigmented patterns can also be used to train the fish to associate with a reward. The initial design also demonstrated that zebrafish can discriminate among vertical and horizontal line patterns (20).

c. Social interaction may also be used as a reward, in which case the reward box containing the fish would be transferred into a tank containing other zebrafish. In this instance, use of fluorescent GloFish® may be advisable so that the subject is not confused with the fish in the social reward tank.

7. Anticipated Results

Due to variability in response among fish, 8–12 replicates for each drug exposure or concentration (or fish strain) may be necessary. The acquisition task will initially take close to the 10 min/trial limit, and some fish may also fail to choose at first. However, at least for food reward for food-deprived fish, the time/trial declined over the 16-day training run to about 20 min/trial. Extinction of an acquired association following these procedures should take over 7 days, and reversal training should take effect more rapidly than the original acquisition (20). The data, which will include average time to complete each trial and % correct/session can be analyzed by repeated measures ANOVA, followed by a parametric post-hoc such as Tukey's HSD or Scheffe's, or a Mann-Whitney U test can be used to compare mean % of correct trials per session. While further validation is necessary, the addition of reward boxes to the T-maze arm ends for color discrimination learning in zebrafish (20) should make the testing protocol more amenable to examining the reinforcing properties of drug reward.

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Chapter 6

Zebrafish Conditioned Place Preference Models of Drug Reinforcement and Relapse to Drug Seeking

Amit Parmar, Miral Parmar, and Caroline H. Brennan

Abstract

This protocol details simple zebrafish conditioned place preference assays for assessing reinforcing properties of drugs of abuse such as ethanol, which can be administered to the tank water. We further describe a conditioned place preference assay of stimulus-induced reinstatement of drug-seeking in zebrafish. The test apparatus consists of a 1.5 L tank that can be divided into two compartments using a Perspex divider and environmental cues that can be placed around the tank. Drug exposure is paired with visual environmental cues at either end of the tank, and a change in preference towards the drug-paired end is taken as indicative of a reinforcing effect of the drug. Using the treatment paradigm described, a 30–50% ethanol-induced change in place preference is observed. Following extinction, this place preference can be reinstated by low-dose, non-contingent ethanol exposure. The simple procedures described here can be used to gain insight into genetic mechanisms contributing to vulnerability to drug dependence and addiction.

Key words: Zebrafish, conditioned place preference, drug reinforcement, relapse, nicotine, ethanol, reinstatement.

1. Introduction

Addiction is a complex psychiatric disorder characterised by a range of compulsive drug-seeking behaviours and a persistent tendency to relapse (return to drug taking) even after prolonged periods of abstinence. Mammalian reinforcement models of drug seeking and relapse to drug-taking have given great insight into the mechanisms underlying the rewarding effects of drugs of abuse as well as the neurobiology of relapse (1). However, development of therapeutics in these models relies heavily on candidate

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gene- and hypothesis-driven approaches. Forward genetic and pharmacological screening techniques, as widely used in zebrafish (e.g. reviewed in (2)), have the potential to rapidly advance our understanding of factors influencing reward and relapse as well as aid the development of novel therapeutics: genes and pathways not previously thought to be involved in a given phenotype may be discovered to have a critical role. With regard to the analysis of drug-associated reward and dependence, zebrafish have been demonstrated to show reinforcement responses to common drugs of abuse (3–6) and to show conditioned place preference that persists in the face of adverse stimuli – a key criteria for the establishment of dependence (5). This coupled with the advantages offered by this species for pharmacological and mutation screening make them an attractive model for the study of genetic factors contributing to sensitivity to the reinforcing properties of abused drugs and vulnerability to relapse.

Here we describe a simple conditioned place preference assay based on that described by Darland and Dowling (4) and Kily et al. (5), which can be used to assess reinforcement responses to drugs of abuse such as ethanol that can be added to the tank water. We further describe a simple conditioned place preference assay of drug-primed reinstatement of drug-seeking in zebrafish. In reinstatement assays animals are trained to associate an activity with receipt of the drug and then undergo 'extinction training' during which the activity no longer elicits the drug reward and behaviour returns to basal levels. Subsequently, the effect of pharmacological and environmental stimuli to reinstate the non-reinforced activity (as a measure of reinstated drug seeking) is determined. Using the method described, our work (7) has shown that ethanol-induced conditioned place preference can be reinstated by low-dose, non-contingent drug exposure as seen in mammalian models. The simple procedures described here can be used to gain insight into genetic mechanisms contributing to vulnerability to drug dependence and addiction.

2. Equipment Setup

2.1. Holding Apparatus/Experimental Apparatus

Fish are maintained in individual 1.5 L tanks (Aquatic Habitats, Apopka, FL, USA) containing 1.5 L of fish water per 20 L: 1.5 g sodium bicarbonate (Sigma Aldrich Dorset UK), 0.16 g calcium sulphate (Sigma Aldrich Dorset UK), 0.36 g marine salt (ZM LTD Winchester, UK) throughout the procedure. The individual tanks are 200×100×100 mm high, which can be divided in half with a Perspex divider and have a lid (to prevent fish jumping out during the course of the experiment). When the divider is

inserted, two compartments each measuring 100×100×100 mm are formed. During housing and conditioning, lids are placed on the tanks to prevent fish escaping. Lids are removed during preference testing to ensure clear view of the fish. Each tank is provided with an insert with a perforated base. When necessary, fish are transported between tanks by lifting the insert out and placing it in the new tank, thus minimising handling stress.

2.2. Visual Cues

We use 0.5 cm vertical black stripes vs. 1.5 cm diameter black spots on a white background as visual cues. These cues are presented by means of a sleeve that is placed over the tank such that each half of the tank is surrounded by either stripes or spots distributed evenly on all sides (*see* **Fig. 6.1**). These sleeves can be easily made by printing on standard word processing paper: Generate a template for each cue using available word processing or drawing software (e.g. Microsoft word). Print a page of the relevant cues and photocopy it onto A3 paper. Cut the A3 pages into strips 110×370 mm. Make folds in the strips 120 from one end and 140 mm from the other end. Stick the strips together so as to generate a rectangular sleeve that can be placed over the tank – *see* **Fig. 6.1**. Paper strips can be laminated before folding to minimise water damage.

2.3. Experimental Room

All conditioning and analysis are performed in a dedicated behavioural room with uniform lighting and neutral decoration. Housing conditions are identical to the fish breeding facility: 28°C, 14 h light:10 h dark cycle.

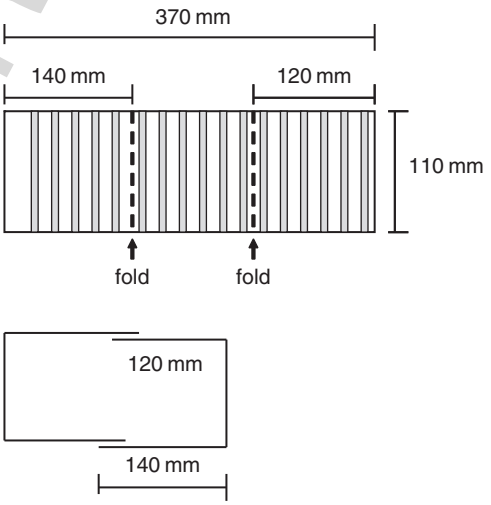

Fig. 6.1. Diagram of environmental cue sleeve.

3. Protocol 1

3.1. Basal Preference

At least 1 day before beginning the procedure, move the fish to be studied to individual tanks in the behavioural room to allow acclimatisation to the new conditions.

Familiarise the fish to the environmental cues and conditioning procedure. This habituation procedure is important to ensure accurate determination of the baseline preference of each individual fish for the environmental cues:

1. Place the fish tank on a sheet of benchkote on the bench (all timing is done on a white background to ensure clear view of the fish, therefore all conditioning should be done under the same conditions).
2. Allow the fish to settle for at least 5 min (although the exact length of the settling period is not critical, the settling period should be the same for all fish).
3. Place the visual cues around the tank and allow the fish to settle for a further 3 min.
4. Restrict the fish to one side of the tank for 20 min using the divider. After 20 min remove the divider and allow the fish to enter the other side of the tank. Restrict the fish to the second side of the tank for a further 20 min.
5. Following the second 20 min restriction transfer the fish to a clean tank in fresh water by lifting the insert and placing it gently into the pre-prepared tank. Fish are transferred to fresh tanks for two reasons; (i) for consistency with conditioning procedure where fish are transferred to fresh tanks to remove them from drug exposure and (ii) so subsequent behaviour is not influenced by possible stress hormones released into the water during the restriction period.
6. Repeat the above procedure each day on three consecutive days. On the fourth day determine the basal preference of each fish being studied.

3.2. Determining Basal Preference

1. Place the tank of the fish to be tested on the bench, remove the lid to allow clear observation.
2. Allow the fish to settle for 5 min, then place the visual cues around the tank and allow the fish to settle once again for a further 3 min.
3. Determine the time spent on a given side of the tank (in the absence of the divider) over a 3 min period. Preference testing can be done manually using a stopwatch or using motion detection software (e.g., Ethovision XT Basic, Noldus Information Technology, Nottingham, UK). In either case care

must be taken that the presence of the observer does not influence the behaviour of the fish – stand well back from the tank and randomise the orientation of the visual cues relative to the observer across the population being tested. The use of tracking software offers a number of advantages over manual observation: Several fish can be assessed at the same time and additional parameters such as mean velocity and distance travelled can be determined. It removes the possibility of observer bias; and if extended time periods are used, once the programme is set up, the observer can leave the room, thus ensuring he/she does not influence the fish behaviour.

4. Determine the basal preference on three separate occasions to ensure it is consistent. The mean of these three assessments is taken as the basal preference. Any fish showing more than 70% preference for one side should not be used further. Note the time each fish spent on each side of the tank – this is their basal preference.

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3.3. Assessing the Reinforcing Property of Ethanol: Conditioning

1. Having determined the basal preference of each fish, separate them into control and treatment groups. (Use at least 15 fish per group.) Place the tanks of the fish to be conditioned on the bench and allow them to settle for at least 5 min.
2. Place the visual cues around the tank and allow the fish to settle for 3 min.
3. Restrict the fish to its preferred side for 20 min.
4. Remove the divider and allow the fish to swim to its least preferred side. Restrict the fish to its least preferred side and add ethanol to give a final concentration of 175 mM (1% vol/vol): 15 ml of 100% ethanol gently added evenly across the tank to avoid the generation of concentration gradients. For control fish, add 15 mL of fish water in place of ethanol. After 20 min transfer the fish to a clean tank with fresh water using the insert.
5. Repeat the conditioning procedure on three consecutive days and then determine the preference of each fish for each side of the tank as described in points 7–9 above. If timing is done manually, ensure that the observer is blind to the treatment condition and basal preference of the fish.
6. Determine the ethanol-induced change in preference by subtracting the time spent on the least preferred side before conditioning from the time spent on the least preferred side after conditioning for each fish. Change in place preference can be expressed in seconds or as a percent of the testing period (as the testing period is 180 s, a 90 s change in preference for the drug-paired side represents a 50% change).

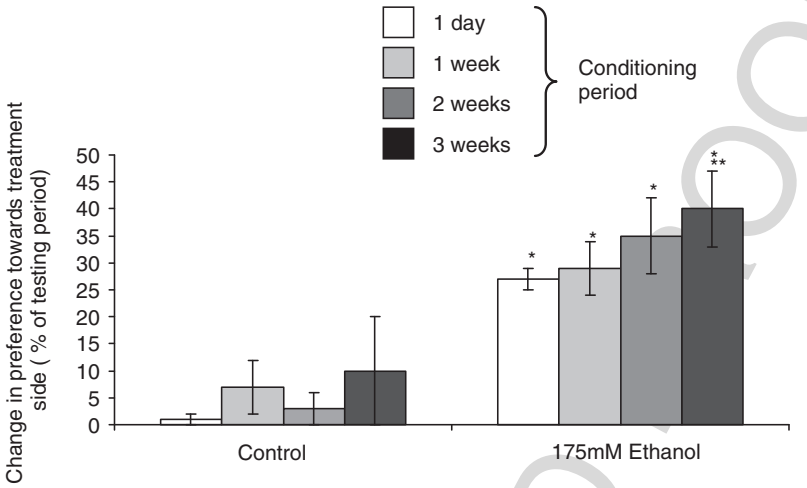

Fig. 6.2. Ethanol-induced conditioned place preference. A single 20 min conditioning session or daily conditioning over a 1–3 week period induces significant change in preference for the treatment side (* $p<0.05$, ANOVA). Conditioned place preference increases as the number of conditioning sessions increases, such that the place preference seen after 3 weeks of conditioning is significantly greater than that seen after either a single exposure or after 1 week of conditioning (** $p<0.05$). Reproduced/adapted with permission from (7).

3.4. Data Analysis/ Anticipated Results

If change in preference is calculated as above, treatment with 175 mM ethanol should give a change in preference of between 35% and 50% for the ethanol-paired side (*see* **Fig. 6.2** for typical result). Control fish are predicted to show a change in preference of up to 10%. Significant differences can be assessed using student *t* test or ANOVA.

NB: The same protocol can be used to assess reinforcing properties of other drugs including nicotine and cocaine (4,5). However, in the case of nicotine particularly, the weight of the fish is an important consideration when determining the concentration of nicotine to be used. We found 20 min exposure to 20 μM nicotine to be reinforcing when 0.5–0.75 g 6 month to 1 year old fish were used. If smaller fish are used, a lower dose is required and the dosage should be normalised according to the weight of the fish (we usually generate a dose-response curve for the individual batch of fish to be analysed).

4. Protocol 2. A Zebrafish Model of Relapse: Reinstatement of Ethanol-Induced Conditioned Place Preference Following Extinction

1. Determine the basal preference for at least 60 fish as described in 1–9 above.
2. Separate the fish into 2 groups of 30 and condition them to either ethanol or saline over a 4 week period: Subject fish to daily conditioning sessions as described in 11–14 above. This number of fish is required to allow for necessary controls to be performed.

3. After 4 weeks of daily conditioning, determine the conditioned place preference for each fish as described in 16 above. Repeat preference analysis on 3 separate days to ensure persistent place preference. This analysis is essential to determine that a robust change in place preference has been induced.

4.1. Extinction Training

1. Transfer the zebrafish from the holding tank into the test tank. Leave the zebrafish to settle for 5 min.
2. Place the visual cues over the test tank and leave the fish in the tank for 20 min without the addition of the dividing panel. During this extinction period, no drugs or control substances (e.g. distilled water) should be administered into the tank.
3. Repeat extinction protocol for each fish once daily for 1–2 weeks to diminish the conditioned place preference.
4. After 1 week take visual cue preference of the zebrafish, as described in point 16 above, to check if extinction has occurred (i.e. the induced conditioned place preference has returned to within 10% of basal). If conditioned place preference no longer persists, repeat the preference test on three separate occasions over the following 3 days to confirm the results and proceed to reinstatement testing. If conditioned place preference remains, repeat steps 1–4 until preference testing shows extinction is successful (up to 2 weeks of extinction training has been necessary in our hands).

4.2. Reinstatement of Conditioned Place Preference Using Non-contingent Exposure to Ethanol

1. Transfer the zebrafish from the holding tank to a fresh tank containing 1.5 L of fish water and allow the zebrafish to acclimatise to the new surrounding for at least 5 min. No visual cues are present at this stage.
2. Add ethanol to give final desired concentration (e.g., 88–175 mM; 0.5 or 1% vol/vol, respectively) and leave the fish for 10 min. Control fish are exposed to saline for 10 min. Four groups of 15 fish should be used: ethanol-conditioned ± drug-priming and saline-conditioned ± drug-priming.
3. Transfer the fish into a clean tank containing 1.5 L of fresh fish water by lifting the insert and placing it into the new tank. Try to minimise transfer of water between the tanks. Allow the fish to acclimatise to the new environment for 5 min.
4. Place the visual cues around the tank and leave the zebrafish to habituate for 3 min.
5. Measure the visual cue preference of the zebrafish over the next 3 min. A return to place preference for the ethanol-conditioned side (calculated as the place preference for the ethanol-paired side following ethanol-priming minus the

post-extinction place preference for the ethanol-paired side) is indicative of drug-primed reinstatement of drug seeking.

4.3. Data Analysis/ Anticipated Result

Conditioning is expected to induce a 35–50% change in preference for the drug-paired side. This ethanol-induced place preference is expected to return to within 10% of basal within 2 weeks of extinction training. In our hands 10 min exposure to 175 mM ethanol induced a 35% change in preference for the ethanol-paired side (*see* **Fig. 6.3**). Change in preference is calculated as time spent on the drug-paired side after treatment minus time spent on the drug-paired side before treatment and is here expressed as a percent of the testing period. Control fish show a less than 10% ethanol-primed change in preference for the saline-paired side. Significant differences can be assessed using student *t* test or ANOVA.

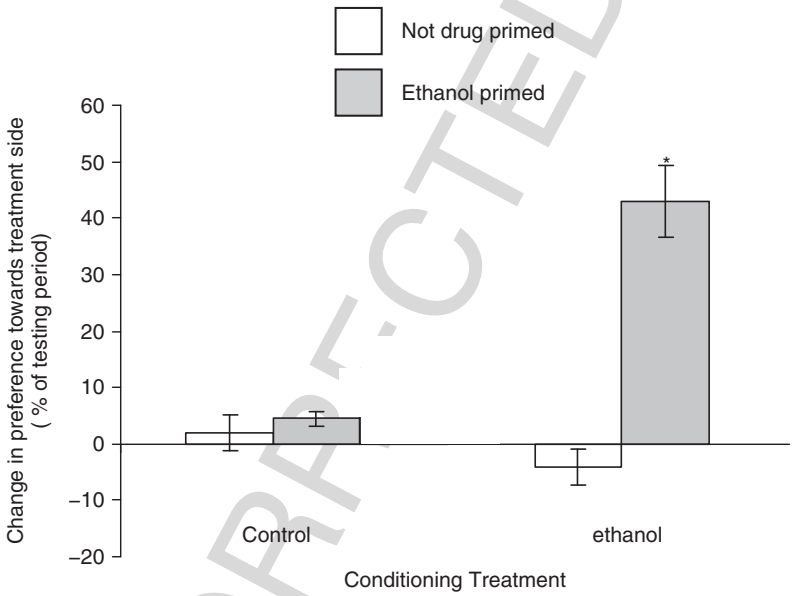

Fig. 6.3. Drug-primed reinstatement of conditioned place preference. Following 4 weeks of daily 20 min exposure to either 1% vol/vol ethanol fish showed a 50% change in preference for the ethanol-paired side (not shown). This preference was extinguished by daily conditioning in the absence of any drug until preference returned to basal and remained there for a 1 week period (not shown). Ten minute exposure to 1% ethanol reinstated the ethanol-induced (*$p<0.05$) conditioned place preference.

5. Trouble Shooting

5.1. Conditioned Place Preference

1. Basal Preference.
   (i) High variance in basal preference after 3 days of habituation

Increase number of habituation sessions from 3 to 5. Minimise human presence and movements (In case of manual measurement of preference, observer should sit/stand still keeping some distance from the test tank so as not to influence fish behaviour because of human presence) near the tank when preference measurement is in progress

(ii) Fish freeze at the bottom of the tank.
This is possibly due to stress. Fish might freeze at the bottom for a time ranging from 1 min to any extended time. In this case, remove the fish to a fresh, clean tank with fresh water. Ensure the temperature of the water is 26–28°C and that the depth of the water is at least 10 cm. Leave the fish to recover for at least an hour and then re-test. If the behaviour persists, do not use the fish further.

(iii) Fish 'peck' at the side of the tank for an extended period.
This may be due to either marks or stickers on the side of the tank, or a reflection of the fish. Minimise labels on the side of the tank. Adjust lighting to minimise reflections. We have tried placing cues on the inside of the tank to prevent reflections.

2. Conditioned place preference.
   (i) High variance leading to no significant change in place preference.
   Minimise the age and weight difference across the population. Increase the number of conditioning sessions from 3 to 5. Increase the number of experimental animals (we routinely use 20 individuals in each treatment group).
3. Reinstatement of conditioned place preference.
   (i) As this procedure relies on an extended period of treatment (conditioning and extinction phases) during which the fish are housed individually, it is essential to ensure proper maintenance of fish water quality and feeding regimes so as to minimise stress. Zebrafish are shoaling fish and do not like being kept isolated for extended periods. Although we house our fish in individual tanks for the duration of these experiments, if stress appears to be a major problem, it may be worth housing the fish in separate chambers within a single tank so that they can sense the presence of other fish (*see* (8)), and then transferring the fish to the test tanks using a net

6. Conclusion

In summary, we describe simple conditioned place preference assays of drug-induced reinforcement and relapse to drug seeking in zebrafish for use with drugs that can be administered to the tank water. The protocols described here give reproducible results in the range shown in both our hands and in other labs (Shannon Saszik, personal communication). These assays advance the use of zebrafish as a model system for the analysis of the neurobiological basis of addiction-related behaviours.

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Chapter 6

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Chapter 7

A Simple and Effective Method to Condition Olfactory Behaviors in Groups of Zebrafish

Oliver R. Braubach, Russell C. Wyeth, Andrew Murray, Alan Fine, and Roger P. Croll

Abstract

We describe a simple assay for studying and conditioning olfactory behaviors of adult zebrafish. The apparatus consists of a circular flow-through tank into which odorants can be administered in a controlled fashion. Odorants (conditioned stimuli; CS) are repeatedly paired with food flakes (unconditioned stimuli; UCS) that are provided inside a tethered floating feeding ring. In response to conditioning, zebrafish develop an odorant-dependent place preference and restrict appetitive swimming behavior to the vicinity of the feeding ring. This robust assay can also be conducted with groups of zebrafish and thus provides a potentially important tool for large behavioral screens.

Key words: Conditioned olfactory behavior, circular flow-through tank, conditioned stimulus, unconditioned stimulus, place preference, appetitive swimming.

1. Introduction

Zebrafish are a favorable model for neurobiological investigations of olfaction. Their olfactory system is representative of that in higher vertebrates, but is reduced in size and complexity. This system is also accessible for physiological study and is easily manipulated by standard genetic approaches. In combination with tractable olfactory behaviors, zebrafish thus constitute a powerful tool for studying the cellular mechanisms that underlie chemosensory behavior and learning. We have recently established an assay for conditioning appetitive olfactory behaviors of adult zebrafish (1). In this chapter we detail how this assay is

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conducted and demonstrate that it can also be used to condition olfactory behaviors through group training.

Upon encountering certain odorants (e.g., an amino acid emanating from a food source), fish initiate appetitive swimming behaviors. These behaviors vary significantly across species (2), but most fish that are used in laboratory settings initiate chemotactic swimming: when fish encounter a decrease in odorant concentration, they will turn to orient themselves towards the direction of increased odorant concentration. This behavior ultimately leads the animal to the source of the odorant (3). We have shown that naïve zebrafish respond to the amino acids L-alanine and L-valine in a similar fashion (1). They increased their swimming behavior and executed more turns (>90°) when compared to normal swimming.

Appetitive chemotactic behaviors can be intensified via positive reinforcement conditioning (4–6). This was first demonstrated in sedentary catfish after repeatedly exposing them to amino acid mixtures paired with food rewards. The catfish ultimately learned to associate the conditioned amino acids with imminent feeding and responded with increased appetitive swimming (3). We have shown that zebrafish also display increased appetitive swimming after olfactory conditioning to both the natural amino acids L-alanine and L-valine, and the neutral odorant phenylethyl alcohol (1). However, appetitive swimming behavior and its modifications through conditioning can be difficult to identify in zebrafish. Zebrafish are naturally active, swimming quickly and displaying frequent directional turns (>90° turns). This activity is often increased during behavioral experiments (due to stress and/or anticipation of reward) and can obscure the detection of appetitive swimming behaviors, which are also characterized by a high frequency of >90° turns. Thus, while appetitive swimming is a useful behavioral measure for work in sedentary species with low levels of normal swimming (i.e., catfish), it may not always be useful for work with active fish species.

To overcome this limitation, we designed an olfactory conditioning method that involves a place preference paradigm. A place preference ensues with repeated positive reinforcement of a set of environmental cues, so that these cues ultimately acquire the motivational properties of the reward (7). We rewarded zebrafish after odorant administrations, and restricted the reward retrieval to the inside of a floating feeding ring. We demonstrated that zebrafish quickly learned to associate this ring with feeding, and that this occurs in an odorant-dependent manner (1). This localized feeding behavior is robust and easily identified, even in highly active fish.

Here we demonstrate that our assay can also be used to condition zebrafish through group training. Our assay is easily conducted, leads to robust olfactory dependent place

conditioning and can be used to train large numbers of fish. These are important criteria for any behavioral assay used for large-scale behavioral screens that are becoming increasingly important in neurobiological investigations seeking to understand genetic and cellular underpinnings of zebrafish behavior.

2. Method

Animals: Our assay can be conducted with zebrafish aged between 2 and 6 months, weighing 0.3–1.0 g. We tested both outbred wild-type zebrafish obtained from a local pet store (AquaCreations, Halifax, NS, Canada) and animals from an established laboratory line (AB strain, University of Oregon). No differences in performance were observed between zebrafish of the different ages or populations listed above.

2.1. Equipment Setup

2.1.1. Tank

The tank is a circular white polypropylene bucket (diameter = 28.5 cm; height = 40 cm) containing a flow-through water system (**Fig. 7.1a**), which provides a rapid, uniform inflow and drainage of the 8 cm-deep water column. The main water inflow (WI in **Fig. 7.1)** is fastened to the vertical wall of the bucket and terminates in a horizontal circular hose, fixed to the bottom of the tank. Regularly spaced (10 cm intervals) holes (I.D. ~ 1 cm) along the underside of this circular hose ensure that the water enters the bucket uniformly. It is important to cover each inflow hole with a mesh (1 mm spacing), because zebrafish will swim into and get trapped inside the inflow tube. As outflow, a polyvinyl chloride standpipe (I.D. ~ 4.5 cm; height = 8 cm) is installed in the middle of the bucket. To ensure that water is drawn off equally from the entire height of the water column, the standpipe needs to be covered with a wider sleeve (I.D. ~ 8 cm; height = 12 cm), in which equally spaced horizontal slits (kerf = 1 mm) are cut at 1 cm intervals. We found it equally important to cover the top of the sleeve, as fish will sometimes jump and may be lost through an uncovered drain.

Odorants are injected via a plastic tube (I.D. = 0.5 cm; see odorant injection tube in **Fig. 7.1a**) that is connected to the main water inflow via a Y-connector. The odorant injection tube is gated by a 3-way Luer valve, to which syringes can be connected (**Fig. 7.1a** inset). The valve needs to be closed when no injections are taking place as the water inflow will draw air into the system

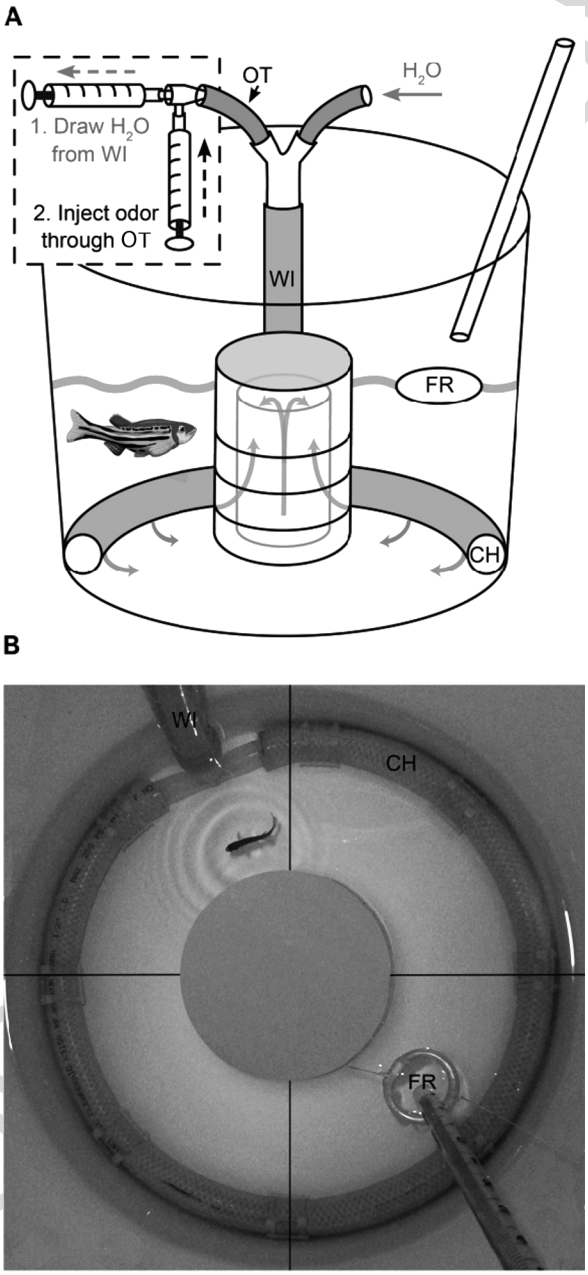

Fig. 7.1. Schematic diagram of the conditioning apparatus (**a**) and still video image (**b**) as recorded from above. Odorants are injected remotely into the main water inflow (WI) and perfuse the bucket through inflow holes spaced along the underside of a circular hose (CH). Following odorant injections, the fish are rewarded inside the feeding ring (FR). The process of injecting odorants is illustrated in the *inset*. To prevent injection of air bubbles into the system, the odorant injection tube (OT) is initially filled by drawing water with a large syringe from the main water inflow (Step 1). Odorants are then injected with a separate odorant syringe (Step 2). The injection tube is rinsed after each trial by drawing water back into the tube (Step 1).

and create large bubbles. To feed the fish, a hollow, plastic feeding tube (I.D. ~1 cm) is mounted to the side of the bucket (above the water level) and aimed at a tethered, floating feeding ring (I.D. = 4 cm; *see* FR in **Fig. 7.1**). We feed the fish with floating food flakes (Nutrafin Staple Fish Food, Hagen Inc., Montreal, QC, Canada), which remain in the lumen of the feeding ring. Both the odorant injection tube(s) and the feeding tube must be sufficiently long for the experimenter to apply both stimuli without being seen by the fish. We also recommend placing the apparatus on high shelves that stand on rubber or styrofoam padding. Zebrafish are very sensitive to vibrations and may respond to the presence of the experimenter rather than odorant injections.

Zebrafish behavior can be monitored and recorded with a standard video camera (30 frames per second) that is placed above the tank. We use a commercially available surveillance video system (Novex Inc., Toronto, ON, Canada) to acquire and view the video clips on our computer. We found it advantageous to have real-time monitoring of the performances of the individual fish and also of the experimenter (e.g., hastened odorant injections may create bubbles that are sensed by the fish). In this way, it is possible to identify potential problems during pilot experiments and prior to conducting lengthy data analyses. Finally, laboratory lighting may be enhanced with fluorescent lights, which should be mounted above the setup. The light is diffused by covering the tanks with white translucent plastic film, leaving only a small hole through which the camera objective can be fit.

2.1.2. Water Flow

Care must be taken to ensure that odorants are administered in a controlled fashion, with predictable onset and clearance. To determine how injected stimuli behave in our apparatus (**Fig. 7.2**), we injected food dye (same volume as odorant injections) into the water inflow and repeatedly drew water samples from the bucket for several minutes. We analyzed the optical density of each dye sample with a spectrophotometer and used these values to create stimulus profiles for each bucket. Using this method, we have determined that injected stimuli are diluted 10^4-fold within 4 min of administration, provided that the volume of the bucket is replaced with fresh water approximately once every minute. We tested a variety of differently sized buckets (0.4–4 l) and the same clearance is achieved in all of these if enough flow is supplied to replace their volume approximately once every minute.

2.1.3. Odorants

The most commonly used appetitive odorants for teleost fish are commercially available L-type amino acids. The amino acid L-alanine (BioChemika > 99.0% purity; Sigma Chemical Co.) is very useful for behavioral work in zebrafish, because it elicits robust appetitive swimming that can also be modified through

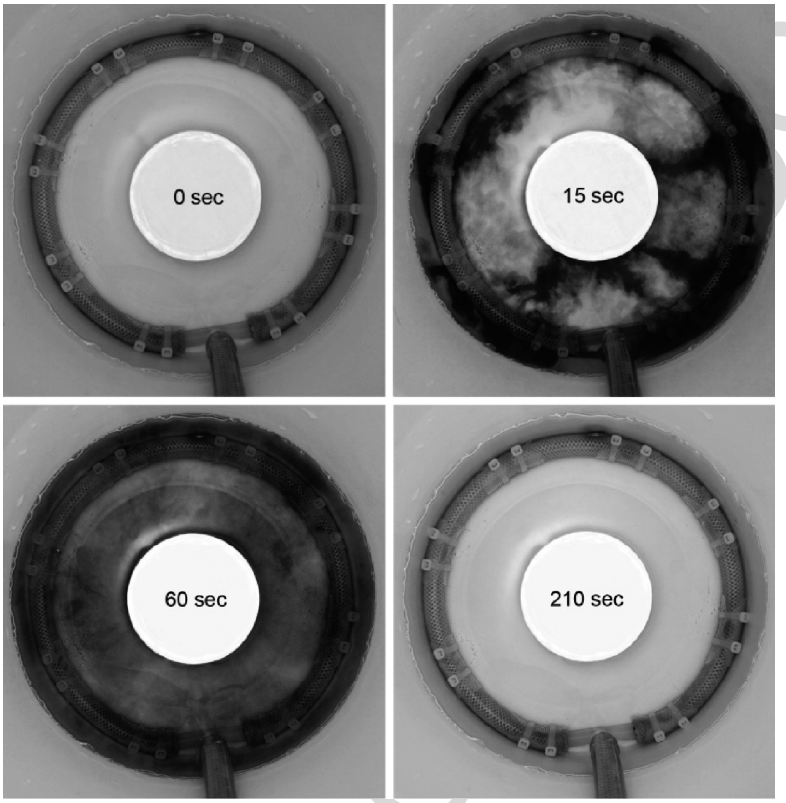

Fig. 7.2. Image series acquired from the conditioning apparatus following injection of 10 mL dye (*black*). The dye quickly spreads through apparatus (15 s) and is evenly distributed within 1 min. A 10^4-fold stimulus clearance is achieved in less than 4 min.

conditioning. As a behaviorally neutral odorant (e.g., conditioned stimulus in classical conditioning), we have used the synthetic fragrance phenylethyl alcohol (PEA; International Flavors and Fragrances Inc.). This odorant does not evoke behaviors in naïve zebrafish, but can be conditioned to elicit appetitive behaviors. Odorants should always be prepared freshly before use and can be injected into the perfusion system as concentrated aliquots. The final stimulus concentration that zebrafish can detect varies widely among odorants, but most amino acids are apparently detected at a final concentration of 10 μM (1,8–10). In all experiments described in this chapter, we used PEA at a final concentration of 1×10^{-4} M as conditioning stimulus.

2.2. Conditioning Procedure

1. Place groups of zebrafish in the buckets. To date, we have trained and tested groups of four individuals of the same sex. Ensure that all fish in a conditioning group are of the same size (*see* **Section 3**).

2. Once the fish are placed in the tanks, adjust the camera and cover the apparatus. Let the fish acclimatize for 24–48 h and do not feed them during this time.
3. We suggest that training be started in the morning, shortly after the light period begins. This will allow sufficient time for conducting all training sessions and ensure that inter-session intervals can be made sufficiently long (*see* 7.).
4. Rinse and prepare the odorant injection tube by drawing water from the main inflow with a large (~ 30 mL) syringe. Close the Luer valve and discard this water. Fill and connect the odorant syringe to the Luer valve (**Fig. 7.1a** inset). Once these steps are completed, record a short video segment (1 min) of the behaving fish. This "baseline" behavior can later be used for comparisons with odorant-evoked behaviors.

   ! Important: Ensure that the odorant injection tube is filled with water prior to injecting odorants. The fish will react to air bubbles that are injected along with the odorant (i.e., through an empty tube).
5. Start olfactory conditioning trials by injecting the odorant (conditioned stimulus) into the water inflow and restarting the video recording. After 45 s [15 s for odorant infusion (**Fig. 7.2**) plus 30 s for behavioral observation], administer food flakes (unconditioned stimulus; a single ~ 2 mg flake for each fish in each trial) through the feeding tube. Watch the fish on the monitor and determine if they retrieve the food rewards and then terminate the recording. In our experiments these are the only feedings that the fish receive. We conduct 12 trials daily and believe that this provides ample food during conditioning.
6. Rinse the injection tube after each trial by drawing water from the main inflow (**Fig. 7.1a** inset). We usually draw enough water to fill a 30 mL syringe and discard this. This ensures that the injection tube is rinsed and prepared for the next trial.
7. Repeat the trial four times during training sessions in the morning, midday, and evening (12 trials per day). Wait at least 15 min between individual trials and 2 h between training sessions. We find that closer spacing of trials and sessions often results in development of odorant-independent place conditioning, where the fish simply remain near the feeding ring in anticipation of feeding.
8. After 4–5 days (48–60 trials), the fish are trained. To determine if each fish within a group has been successfully

conditioned, it is necessary to individually test their performance in the conditioned task. Divide the group and place each fish by itself in a separate conditioning tank. Let the fish acclimatize for 24–48 h and feed them daily, but not through the feeding ring.

9. Prior to testing, we conduct 1 "refresher" trial with individual fish. These trials are conducted in the same manner as training trials and may be necessary for the fish to acclimatize fully to being isolated in the apparatus. A single training trial does not induce an odorant-dependent place preference in individually trained zebrafish, and we therefore believe that the "refresher trial" does not produce conditioned behaviors observed in tests. Conduct this trial at least 1 h before testing the fish.
10. The final test consists of four trials conducted with individual fish to determine if they respond to the odorants with conditioned behaviors. Each trial is performed in an identical manner to the training trials described above, but no food is given to the fish following odorant injections. Perform each probe trial individually, separated by approximately 2 h intervals to minimize habituation to the (now unrewarded) odorants.
11. Before placing new fish into the apparatus, inject household bleach (5.25% sodium hypochlorite) into the system via the odorant injection tube and then turn off the water for 30 min. Rinse well (overnight). This will clean the apparatus and any odors or debris left from the previous training session.

2.3. Control Experiments

To test if olfactory place conditioning is dependent on the specific pairing of odorants and food, these stimuli can be administered independently of each other. For this chapter, we exposed the fish to the odorant (PEA) 12 times daily, on the same schedule as conditioning would normally occur, but we did not feed the fish during these trials. Instead we fed the fish (through the feeding ring) at various times during inter-session intervals. In previous experiments we also assessed the possible involvement of mechanosensation (i.e., the sensing of volume displacement from odorant injections during trials) and gustation in producing learned behaviors (1).

3. Trouble shooting

1. In our apparatus a circular inflow hose is installed in the behavioral arena (**Fig. 7.1a**). It is not uncommon for a fish to be initially hidden beneath this inflow tube. Typically, odorant infusions are enough to lure the fish out of hiding (i.e., they respond with swimming activity), but if this is not the case, we suggest insertion of a mesh barrier into the behavioral arena. We have built such barriers with 1 mm Nitex mesh and these effectively prevented the fish from accessing the tubing (not used in the experiments described here). It is best to make the barriers removable, because the mesh traps debris and requires cleaning after experiments.
2. One or several fish in a group may become stressed in the apparatus and this can affect performance during conditioning. Stress may manifest itself in several ways. The fish may swim very quickly and repeatedly around the circumference of the apparatus (circling). If fish do this continuously for a day after acclimation, they will continue to circle the apparatus and will not respond to training. Alternatively, stressed fish may hide under the inflow hose (if there is no mesh to restrict access) and remain there for the duration of the experiment (without visibly responding to odorants). As with the circling behavior, fish that remain under the tube for a day after acclimation will generally not be useful for conditioning. It is thus important to check for these and other behaviors after the acclimation period. If necessary, replace the stressed fish and let the group acclimate for another day before starting the conditioning experiment. In our experience, fish that are not "stressed" after acclimation will not become stressed during conditioning; nevertheless, we recommend continuous monitoring for any signs of stress.
3. Some fish fail to retrieve the food reward at the end of a training session. This is not uncommon, especially in a group where competition for food exists (*see* also below). If a fish does not retrieve the food reward or approach the feeding ring for a whole day of training (due to stress or competition), it may not be conditioned adequately. It is important to be aware of such individuals during data analysis. If the fish are individually identifiable, it may be helpful to remove the fish in question and continue training the remainder of the group.

4. During training, when the fish are conditioned multiple times in quick succession, it is common that they develop a nonspecific place preference and the feeding ring regardless of the presence of an odorant (1). This place preference becomes more robust as successive rewards are administered more rapidly. To ensure that fish develop an *odorant-dependent* place preference, it is therefore very important that inter-trial intervals are sufficiently long (minimally 15 min). This permits the fish to return to baseline behavior after each trial and impairs the development of a nonspecific place preference. In preliminary experiments we have found that longer spacing of training trials (one trial every ~ 45 min) prevents the development of a nonspecific place preference, but not the odorant-dependent place preference (unpublished observations).
5. Finally, in group-training experiments it is important that all fish in a group are similarly sized. We have repeatedly observed larger fish in a group apparently displaying territorial dominance near the feeding ring. This prevented smaller fish from obtaining the food reward and likely affected their acquisition of conditioned behaviors. Similarly, we noticed that groups of fish obtained from the same holding tanks (provided that they were the same size) readily shoal with one another, while groups of fish from different holding tanks (i.e., different families and ages) were more aggressive towards one another. Even after meticulously selecting animals for our group training experiments, we found considerable variability in the way that fish behaved as a group. We therefore suggest careful observation of the animals during training and to be aware that some individuals may not learn the task due to dominance of other fish.

4. Analysis

To determine if individual zebrafish develop a place preference following group training, we test each fish individually and measure the time that it spends in the area of the bucket containing the feeding ring. We divide the total area of the bucket into four quadrants (**Fig. 7.1b**) by placing a grid drawn onto acetate sheets onto the computer screen. The time that fish spend in each quadrant can then be recorded with a stopwatch or appropriate video analysis software (11). Fish that are distributed at chance will spend 25% of the observation period in each of the four quadrants. A place preference to any quadrant then manifests

itself as an increase in the time that a fish spends in a single quadrant (*see* below). Conditioning can also lead to changes in appetitive swimming behaviors (i.e., frequency of >90° turns) and changes in swimming speed. We have scored such changes manually (1), but suggest that future experiments take advantage of more sophisticated and practical computerized behavioral analysis (11).

Data derived from this experiment will consist of repeated measures of the performance of individual fish during training and testing. An appropriate analysis will thus employ a repeated measures analysis of variance to identify changes that occur within and between treatment groups. Within group effects (or a regression analysis) can be used to identify temporal effects of conditioning [i.e., acquisition curves; *see* (11) for acquisition data for group training], while between group effects will reveal if there are any differences in performance between experimental groups. Finally, we found that performance of individual fish is prone to substantial variability between trials, and we therefore use the mean performance of fish in training sessions (mean of four trials) as data to analyze the effects of conditioning.

5. Results and Conclusion

Group-trained zebrafish show a place preference to the quadrant containing the feeding ring (**Fig. 7.3**). In response to the conditioned odorant PEA, individually tested fish ($n = 12$) spent 45.5 ± 1.7% of the test duration (30 s) in the reward quadrant containing the feeding ring. This was significantly increased from the time spent in the reward quadrant prior to odorant administration (27.5 ± 2.5%; repeated measures ANOVA: $p < 0.001$). The conditioned zebrafish also spent significantly more time in the reward quadrant than fish in the control group that were repeatedly exposed to PEA without subsequent food rewards (*see* Control Fish in **Fig. 7.3a**; between subjects effect repeated measures ANOVA: $p < 0.05$). These data thus indicate that group-trained zebrafish respond to the odorant by localizing to the reward quadrant and that this behavior develops as a result of pairing PEA with rewards administered in the feeding ring.

To summarize, our behavioral assay relies upon an inexpensive apparatus, is easily conducted, and is adaptable for use with large numbers of animals. It therefore meets the requirements of many laboratories and could emerge as a popular tool for behavioral research of the olfactory system. In closing, we suggest that any

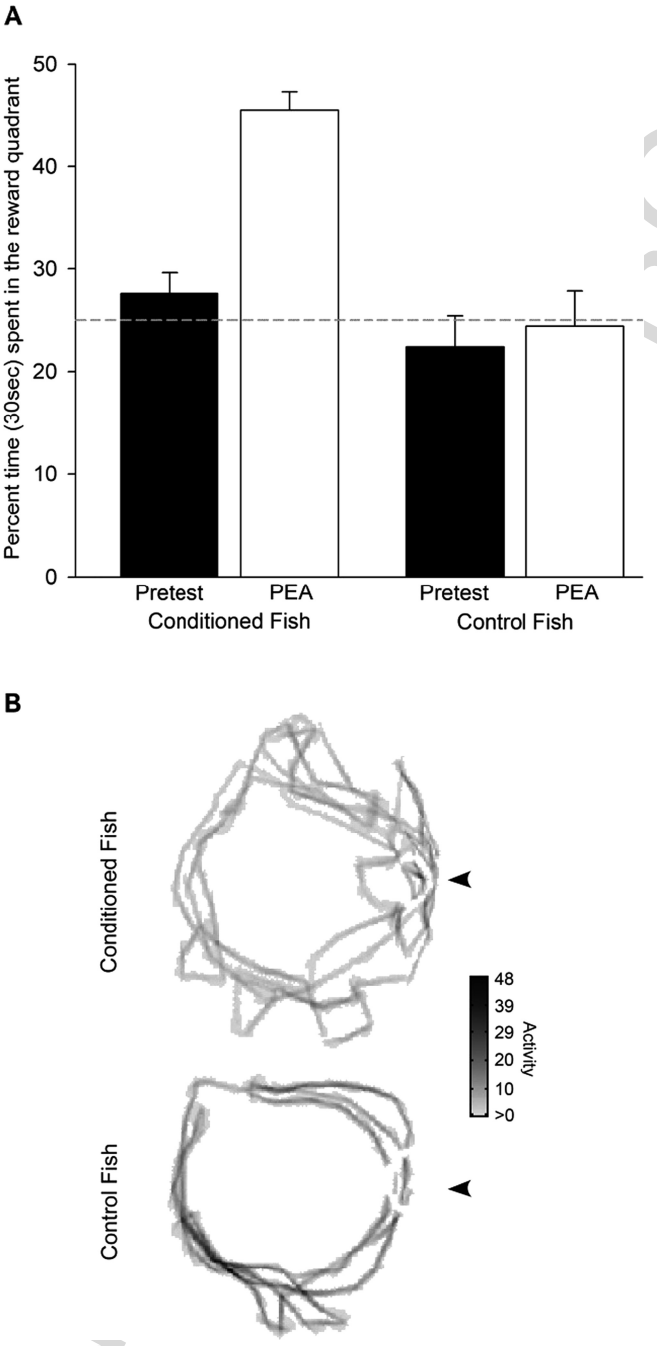

Fig. 7.3. (**a**) Group-trained zebrafish localize to the reward quadrant when exposed to the conditioned odor PEA during individual testing (conditioned fish). Prior to each test, the fish are distributed at chance throughout all areas of the apparatus, indicating that the place preference for the reward quadrant is odorant-dependent. Control fish that received PEA only and were fed at other times during training did not develop a place preference. All data shown in (**a**) are the mean scores from 12 fish tested over four trials and their standard errors. The dashed grey line (25%) indicates chance distribution. (**b**) The distribution of two individual fish during testing is shown in videograms.Conditioned fish moved faster and returned to the ring more often when exposed to PEA. Control fish moved slowly and showed no place conditioning. The videograms were mapped onto a common coordinate system with the same feeding ring location (*arrowhead*). Activity scale: activity frequency over 30 s, sampled at 30 frames s–1 (for more details, *see* (11)).

other small teleost (e.g., medaka, goldfish) could be tested equally well for basic odorant responses (1) and cognitive capabilities through our method.

Acknowledgments

We thank Erika Harding for help with the group training experiments, and Evan McCarvill for the zebrafish illustration in **Fig. 7.1a**. This work was supported by research grants from the Nova Scotia Health Research Foundation (to O.B), the Malacological Society of London (R.W), the Canadian Institutes of Health Research (to A.F) and the Natural Sciences and Engineering Research Council of Canada (to R.P.C).

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Chapter 8

Aquatic Light/Dark Plus Maze Novel Environment for Assessing Anxious Versus Exploratory Behavior in Zebrafish (Danio rerio) and Other Small Teleost Fish

Georgianna G. Gould

Abstract

Initial introduction into novel environments produces in zebrafish behaviors consistent with predator evasion, fear, and/or anxiety. This protocol is for a light/dark plus maze novel environment test utilizing two elements of those behavioral responses, thigmotaxis and light avoidance, to assess "anxiety states" in zebrafish. The test is based upon the rodent elevated plus maze and is scored similarly, except for fish white arms replace opened arms, and black arms parallel closed arms. Several pharmaceuticals that reduce anxiety levels in patients and increase open arm exploration by rodents in the elevated plus maze also increase zebrafish entries into and time spent in white arms of the maze.

Key words: Novel environment, light/dark plus maze, thigmotaxis, light avoidance, exploration, anxiety.

1. Introduction

Zebrafish (*Danio rerio*) behavioral assays focused on innate responses holding predictive, face, and construct validity with human anxiety disorders are in demand for translational biomedical research. One approach to modeling anxiety in zebrafish is to introduce individual fish into novel environments and observe their immediate response. Individual zebrafish newly introduced to an environment exhibit thigmotaxis, touching, or lingering near the bottom and sides of the aquarium for several minutes. This response has been attributed to anxiety or avoidance of predation associated with the novel tank environment (1–3). Thigmotaxis is short-lived, so after 3–5 min fish begin to explore the new environment more extensively. Acute

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exposure to ethanol, nicotine, benzodiazepines, and other anxiolytic compounds reduces the duration of thigmotaxis (1–5). Finally, the duration of thigmotaxis depends on fish naïveté, as zebrafish introduced into tanks of the same dimensions of their housing tanks exhibit significant reductions in time spent bottom dwelling (4).

A second way to generate anxiety in zebrafish is to alter the intensity of lighting or make the background of a novel test tank white. Zebrafish tend to seek dark backgrounds (or avoid light backgrounds) in unfamiliar environments (6, 7). When zebrafish are exposed to bright light or placed in habitats with white or light-shaded backgrounds, the transient early neural induction factor *c-fos* is expressed in the dorsalmedial telencephalon (comparable to mammalian amygdala) to a similar extent as when fish are shaken vigorously in a dip net, or exposed to a convulsant agent, indicating that such stimuli evoke neural activity comparable to a mammalian fear response (unpublished work, 8). This response can be attenuated by prior acute treatment with anxiolytics.

My laboratory has developed an alternative test, the aquatic light/dark plus maze for zebrafish (and other small teleosts), to measure early fish behavioral response to a novel environment. Overhead lighting in a new environment evokes an "anxiety-like state" for the subject fish, which is presented with a choice of white or black background. The aquatic light/dark plus-maze is derived from the rodent elevated plus-maze anxiety test, with black arms analogous to closed arms and white arms to open arms. Fish behavior is scored in a similar manner to the scoring of rodent behavior in the elevated plus maze (9). The number of crosses into white or black covered arms or the middle square is tallied, and the time spent in white arms versus other parts of the maze is recorded. We also measure time spent frozen in the dark grey-shaded middle square, or latency to move, which is particularly evident in fish administered high doses of drugs with sedative or neurotoxic properties. We have not attempted to quantify thigmotaxis in the maze, but we have observed it; and certainly through real-time observation or a strategically placed camera, this dimension of behavior could be measured as well.

The aquatic light/dark plus maze can be used alone or in conjunction with the novel dive tank (3) to measure the temporal dynamics of defensive versus exploratory behavior. We have found the behavioral response to be sensitive to acute and chronic dietary treatments with anxiolytic or anxiogenic compounds (5, 10). In the aquatic plus maze, to date, we have used predominantly adult zebrafish >90 days old, with body lengths ranging from 2 to 4 cm (11). Our pilot tests with juveniles in the plus maze have been less extensive, but it appears that the full size maze may also be amenable to testing 60–90 day old (1–2 cm long (11)) zebrafish since they exhibit similar behavioral

patterns to adults. We have also observed adult behavior of other small fish, such as goldfish (*Carassius auratus*) or fathead minnows (*Pimephales promelas*) in the maze, and both exhibit similar initial avoidance of white-background and arm entry behaviors. Finally, as there is much interest in examining larval zebrafish behavior for drug and toxicological screening, we are developing a half-scale light/dark aquatic plus maze for use with larval fish.

Ideally, it would be best if the experimenter(s) can leave the room and instead videorecord fish behavior in the aquatic light/dark plus maze, scoring the results by viewing the recordings afterward. If this is not possible, and researchers must score the test in real time by eye and hand, it is best if two people can observe and score together so that one person can tally line crosses and the second can operate the timers, record initial latency to move from the maze center, and time spent in white arms. We have been observing and scoring the behavior while standing approximately 90 cm from the maze, which sits on a 107-cm high benchtop. Further, behavioral analysis software such as Noldus Ethovison®, Stoelting ANY-maze™ or equivalent behavioral tracking and monitoring software may be used to develop an ethogram or track the order and timing of visits to each arm of the maze.

2. Materials

1. Several 500 mL–1 l beakers filled with habitat water (for acute drug exposures, controls, and holding fish after drug treatment or prior to testing)
2. Offset cross maze (Ezra Scientific, San Antonio TX, *see* **Section 2.1**)
3. Conditioned and warmed habitat water
4. Zebrafish for testing (sample sizes from 6 to 10 are recommended)
5. Drug or test compound of interest (effective concentrations for most acute aquatic exposures fall in the range of 1–100 mg/L)
6. Solvent such as DMSO, if required
7. Two or more digital timers, for use in real time or subsequent scoring from video (one to score total run time, the others to record time spent in white versus black arms, and in the middle zone)
8. Digital camera(s)
9. Software such as Stoelting Anymaze™ or Ethovision® by Noldus, if desired

10. Index cards for hand or video scoring
11. Black and white (matte, not shiny) polypropylene binders or folders, cut to fit inside arms of maze (eight 10 × 10 cm panels of each)
12. Dark grey background with white 1 cm square grid for center square, printed on laser or ink-jet printer from PowerPoint (Create a slide with grey background by selecting solid fill black, text 1, lighter 35%, then impose on it a table grid of white at least 14 rows × 14 columns with 1 cm^2 squares with no fill.)
13. Medium-sized binder clips, 12
14. Copy stand to mount camera above maze (Kaiser RS1 or equivalent)
15. 60 W incandescent light bulb (perched above and behind camera)
16. Dip nets, 1–2 (additional nets required for each drug exposure to reduce cross contamination, or rinse nets between treatment)

2.1. Equipment Setup

The offset cross maze, commercially available from Ezra Scientific (San Antonio, TX), is configured to be used as both a T-maze for conditioned place preference as well as a cross maze for the aquatic light/dark plus maze, hence its distinctive shape (**Fig. 8.1**). The 71 cm high × 51 cm wide offset cross maze is constructed of clear 0.32 cm acrylic sealed with aquarium sealant, and is subdivided into 10 × 10 cm (10 cm^2) modules by drop in doors. For light/dark plus maze tests, the offset cross maze is configured in a module with a 10 cm^2 center section as the starting place for the fish, and the four adjacent arms

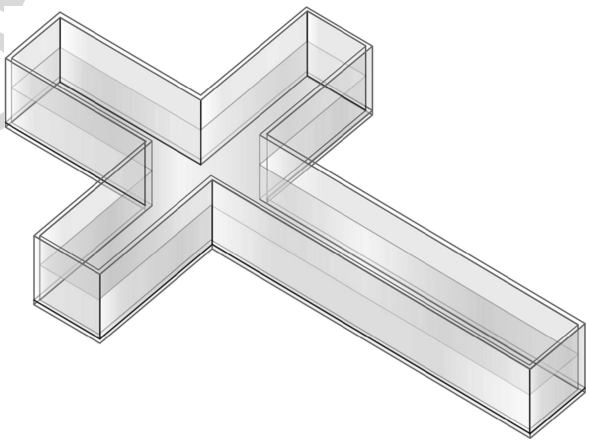

Fig. 8.1. Diagram of the offset cross maze. Maze ends are comprised of 10 × 10 cm squares, side and top arms are 20 × 10 cm, the bottom long arm is 40 × 10 cm.

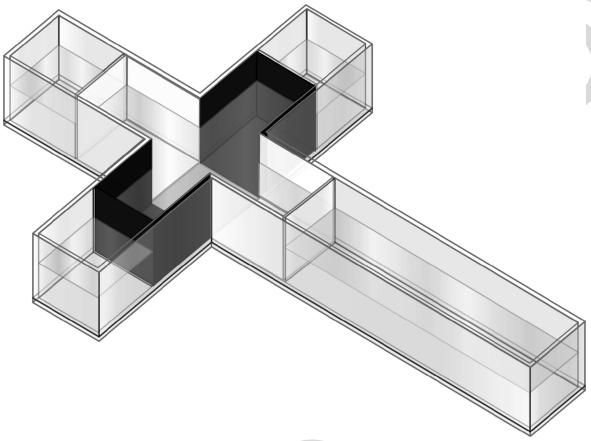

Fig. 8.2. Diagram of offset cross maze configured for use as an aquatic light/dark plus maze. Each 10 cm^2 section off of the center is sealed off with a drop in door, and lined with either *black* or *white* poly squares.

consisting of two additional 10 cm^2 sections opened, and all other sections closed off by sliding doors (**Fig. 8.2**). The maze is 10 cm deep, and should be filled to a uniform depth of 5 cm with 3.5 l of home tank water. Two opposite arm bottoms and sides are lined with black polypropylene and the other two opposing arms are lined with white polypropylene sections cut from file folders (Office Max, USA). Poly sections should be clipped to the insides with binder clips and submerged on the bottom of the maze after water is added (**Fig. 8.3**). We have tried covering the outside of

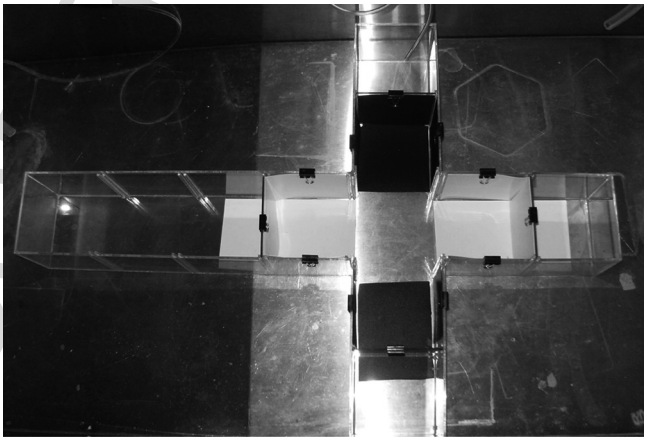

Fig. 8.3. Photograph from above of aquatic light/dark plus maze configuration of offset cross maze. When behavior is being tested, the middle square is placed on *top* of a copy stand that is 35% lighter than *black*. If such a copy stand is not available, a similar *grey* background can be printed out as a slide from Microsoft PowerPoint on paper to be placed under the maze. The maze in this photo had no water inside and was placed in a stainless steel sink to better demonstrate the position of poly linings and clips to the inside of arms.

the maze with the black and white squares, but glare from the walls and bottom of the maze seem to interfere with the fish's perception. It is better to affix the black and white poly squares to the inside of the maze so fish may observe and contact them directly. The middle square is left uncovered (fish may perceive glare from the acrylic surface). The maze is then placed on a copy stand (Kaiser RS1, B&H Photo, New York, NY), which has a grey background with white 1-cm square gridlines showing through the middle square and a digital camera is mounted on the stand. In lieu of the Kaiser RS1 grey copy stand, a grey background that is 35% lighter than black with white 1 cm (2) gridlines can be made in Microsoft PowerPoint and printed to put *under* the maze (*see* **Section 2**). A lit 60 W desk lamp should be situated on the copy stand above the maze and behind the digital camera (HP Photosmart R742) during testing, and adjusted to an angle that reduces glare to the camera or observer(s).

2.2. Acute Drug Exposures

Our zebrafish are generally housed in groups of 6 in the 3 L tanks of a benchtop aquatic habitat (Aquatic Eco-Systems, Apopka, FL) with recirculating filtered deionized tap water, 27°C, supplemented with 200 mg/L Instant Ocean® synthetic sea salts (Spectrum, Atlanta, GA), henceforth referred to as "habitat water". Housing conditions may differ among labs; however, it is important that all zebrafish being compared within an experiment and between treatment groups are housed under the same conditions in tanks of uniform size. Zebrafish are exposed to drug in 250–300 mL of habitat water in a 600 mL beaker. Acute bath exposure duration to water-soluble drugs or chemicals is typically 3–5 min in our laboratory. Using [^{3}H] citalopram (79 Ci/mmol, Perkin-Elmer, Boston, MA) bath exposures in the μg/L range for 3 min, we approximated by linear regression that exposure to 100 mg/L of citalopram and similar compounds should result in concentrations near 100 μg/g in brain and 1000 μg/g in muscle (5). Solvents such as DMSO, acetone, or ethanol can be used at low concentrations, but it is important to run a vehicle control group of animals since such solvents can also produce increased mobility and may trigger more frequent visits to white arms. Other approaches for administering insoluble compounds include subchronic dietary exposure in gelatin food mix (10), which has worked well in my lab, or injection (12), which we have not tried since restrained and injured fish might behave differently in the maze.

During drug exposure, it is important to watch zebrafish carefully or cover the exposure beaker with breathable cotton gauze and secure with a rubber band, as they have a tendency to jump from these vessels. After acute exposure, zebrafish should be transferred to a 1 L beaker for 5 additional minutes for the drugs to take effect. During this time we have often interjected the dive

tank test (3), and have observed decreased bottom dwelling in response to the administration of anxiolytic drugs and other compounds immediately following 3 min of drug exposure (5). Our selection of exposure and holding times is based on results we have obtained for use of benzodiazepines and reuptake inhibitors, and is intended as a preliminary guideline. Pilot studies should be conducted for any new compound to determine onset and offset of maximal response.

3. Procedure

(1) First, prepare a bath solution of drug or compound for exposure of fish (typically we dissolve drugs in 250–300 mL of habitat water) in a 600 mL beaker. Smaller volumes can be used for more expensive or rare compounds. Prepare a second bath for controls containing an equal volume of just habitat water. When using a solvent such as DMSO, a vehicle control exposure should be prepared in addition to the habitat water control. For fish administered drugs or compounds chronically by diet (10) or via injection (12), ensure comparable treatment of controls and proceed to step 3.

CAUTION! If injections are the only successful mode of administration for a drug or compound, it is essential to also inject a set or subset of control fish with vehicle fluid, as the injection process alone may alter behavior.

(2) Take a fish with a dip net from the population being tested and add it to the drug bath solution for 3–5 min.

CAUTION! Do not over expose fish to high concentrations of drug (in the 100 mg/L range), fish may become immobilized or perish. Maintain the same exposure times for all fish in the experiment, so this does not become a confounding factor.

(3) Remove fish from exposure beaker and transfer to holding beaker for 5 min with dip net.

(4) While exposing fish to drugs and the holding tank, set up camera, light, and fill aquatic light/dark plus maze with habitat water.

CAUTION! Be sure that the camera is sufficiently high above the maze and panned such that both white and black arms will be completely visible. Assess glare level and adjust the overhead 60 W light to reduce it.

(5) Attach white and black poly squares to the bottom and sides of the maze.

CAUTION! Press and smooth the poly squares lying at the bottom to remove any trapped air bubbles to ensure that they do not float up. Also be certain that there are no gaps between the poly squares and the sides of the maze. Zebrafish will swim under the bottom or side poly squares and get stuck if the opportunity presents itself.

(6) Place the maze on the grey copy stand with white gridlines, or on the PowerPoint grey background printout so that it shows through the middle square of the maze.

(7) If observing and scoring by hand, prepare an index card by drawing a 2-D schematic of the zebrafish maze boxes for tally marks to be recorded as the fish crosses into each arm. If videotaping, it is ideal to score the behavior afterward so fish are not distracted by the observers.

CAUTION! If you rely on video-recording the behavior to score later, make sure the camera is on and has sufficient battery life before retreating from the room, a backup camera at a slightly different angle can further prevent data loss.

(8) After 5 min in the holding beaker have transpired, turn on the videocamera and begin the timer when fish are transferred via dip net into the center square of the aquatic plus maze.

CAUTION! Do not remove fish from water for more than several seconds, the time it takes to smoothly transfer them from the holding beaker to the maze. So long as they are submerged under the water in the center square, fish can be held within the dip net as the camera is turned on and a card with number or name identifying the fish is shown to the camera. Then release the fish from the net into the center square of the maze.

(9) If scoring by hand in real time, tally all crosses into black and white arms as well as in the middle section. Record time spent initially frozen in the middle, and time spent in white arms.

CAUTION! To minimize distraction of fish, it is critical that the observers are silent, motionless, and not readily visible to the fish.

(10) After 5 min of testing is complete, turn off the video camera and remove fish from tank with a dip net. It may be of interest to weigh the fish at this point, but since this can be a stressful procedure, it is advisable to wait until after

the test to do so. Transfer zebrafish to a separate home tank (we use 1 L tanks in our habitat and label each for easy identification).

(10) Empty water from the aquatic plus maze and rinse maze and poly squares with deionized water.

CAUTION! Re-use of habitat water filling the plus maze is not advisable, as fish may leave odors in the water that can be perceived by other fish.

(11) This procedure (steps 1–10) can be repeated as many times as necessary to incorporate a number of different drug treatments or concentrations of drugs randomly interspersed with controls. We conduct these tests during the normal light phase of the light-dark cycle.

CAUTION! Do not re-use fish, it is essential to the experiment that the fish be naïve to the maze.

4. Anticipated Results

After obtaining 6–10 replicates for each drug exposure or concentration (or fish strain) of interest, results can be analyzed parametrically. The goal is to compare the means for the fish populations tested for the parameters of: total line crosses, % white over total line crosses, time spent in white arms and time spent frozen in the middle. It is best to analyze the data in units of seconds. As several dependent variables are generated that are interrelated, a multivariate analysis of variance MANOVA (one or two way for drug or drug × dose) can be used to analyze the data, followed by ANOVA and Tukey's or Fisher's LSD (or equivalent tests) for post-hoc analysis of significant results. Data can be presented as bar graphs of means with standard errors shown for all four parameters, or just the parameters for which significant results are evident. This novel light/dark plus maze test is a useful screen for anxiolytic drug properties and can also be used to enhance research of evolutionary origins, developmental processes and neural pathways involved in fear and anxiety.

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Chapter 9

A Novel Test Battery to Assess Drug-Induced Changes in Zebrafish Social Behavior

David J. Echevarria, Christine Buske, Christina N. Toms, and David J. Jouandot

Abstract

The zebrafish (*Danio rerio*) has been at the forefront of neurobiological research and is steadily gaining favor as a model organism for behavioral applications. The ease of handling, high yield of progeny, and efficient mode of drug delivery make this species a particularly useful model for behavioral screening. Various drug classes have a range of physiological effects that can impact motor output and social behavior. Using a behavioral paradigm in drug screening can be a useful tool, from determining dose ranges and toxicity to drug-induced behavioral changes. Here we propose a novel behavioral paradigm to assess the group dynamics of zebrafish. This protocol describes methods for simple, fast, and accurate assessment of drug-induced effects on motor and social behavior.

Key words: Motor behavior, social behavior, shoaling, circling behavior, drug screen, thigmotaxis.

1. Introduction

Zebrafish show a natural tendency to shoal (1). Utilizing this feature, shoaling behavior can be a powerful tool in the assessment of drug effects, particularly on the brain. A large number of human conditions and diseases involve a behavioral component, such as chronic anxiety, alcoholism, depression, Alzheimer's disease, and autism (2–5). The continuous effort to improve on and develop new treatments for these, and other conditions, secures a continued need for reliable drug testing methods and models. There have been an increasing number of research investigations highlighting the behavioral spectrum of the zebrafish and drug challenges (4). The easy of handling, high yield of progeny, and

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efficient mode of drug delivery make this species a particularly useful model for behavior. Because the zebrafish model affords an alternative and efficient mode of drug delivery via the gills, submersion is the primary method used (3, 6, 7).

The zebrafish shares more similar features to humans than other genetically homologous models, such as *Drosophila melanogaster* (8). Many features of the zebrafish make it a particularly attractive candidate for inferring higher-level vertebrate behavior, and thus a suitable behavioral model organism. The anatomical similarities of neurotransmitter pathways, like dopamine, may indicate comparable neural functionality to other vertebrates (5). Zebrafish central nervous system development closely resembles that of other vertebrates and has been the focus of most research thus far (9). The nervous system structure of this particular species of fish has allowed researchers to draw conclusions regarding the function of the human nervous system. Recently, commercial resources (e.g., Zebrafish Information Resource Center, ZIRC) and the availability of selective genetic progeny (10) make research on the zebrafish an efficient and inexpensive addition to behavioral inquiries.

Zebrafish are a shoaling fish with an elaborate behavioral repertoire, including but not limited to, well-defined subordinate and dominate roles. Shoaling behavior is best captured in the context of group cohesion, which has shown to be dynamic and complex (11). Swim behavior, and likely shoaling behavior, can be manipulated with dopaminergic modulations (12). Dopamine agonists (e.g., SKF 38393) elicited a variety of behavioral responses in the rodent model including hyperactivity and increased locomotion (13). Taken with the above evidence, it is highly plausible that zebrafish would exhibit a similar behavioral repertoire to pharmacological exposure. Put another way, the known effects of the dopamine agonist SKF 38393 results in hyperactivity and increased locomotion in the rat. If individual zebrafish exposed to SKF38393 displayed hyperactivity and increased locomotion the net result should be the disruption of the group behavior, shoaling. Group cohesion, normally the result of individual interactions (i.e., dominant and subordinate hierarchies), would become disrupted.

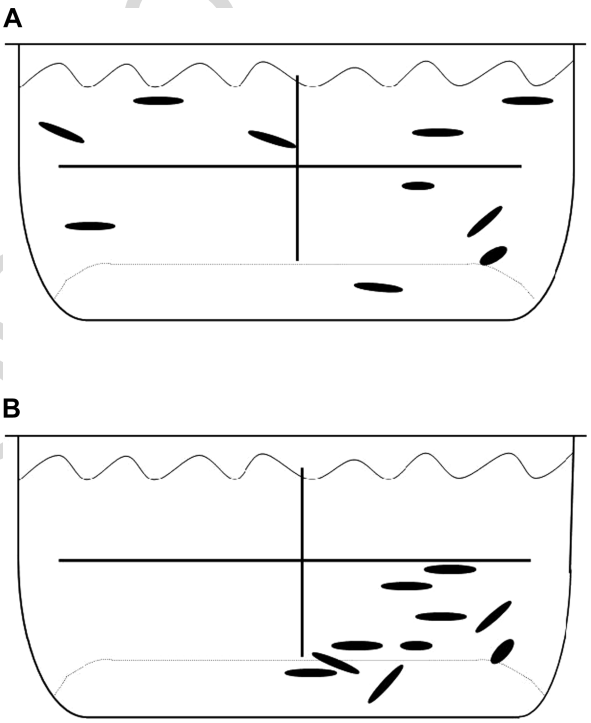
Shoaling behavior, when tested in a group context, provides us with a different insight into social display that compliments data captured from other behavioral paradigms in which one subject is tested at a time. This protocol hinges on this principle and introduces a novel method of testing shoaling behavior in zebrafish, using an open field paradigm. To determine group dynamics, a grid system is used allowing the quantification of zebrafish present in the same relative area over time. The current sets of experiments were designed to extend on the existing research regarding zebrafish behavioral assessment.

2. Materials

Adult zebrafish must be experimentally naïve, and acclimatized to their environment for at least 10 days prior to testing. Zebrafish should be housed in a community tank system at a temperature of approximately 27°C. Illumination should consist of ceiling mounted fluorescent lights, which are kept on a regular dark light cycle (e.g., 14 h on and 10 h off). The tank system must contain aeration and filtration systems, and the water must be dechlorinated for housing and testing tanks.

Behavioral recording is done with a mounted video camera. This protocol consists of two main parts, each requiring separate testing environments:

The Shoaling Paradigm requires a plexi-glass testing arena. Suggested dimensions are: 13×18×29 cm. Using an arena of these dimensions requires a water volume of 5,000 mL. However, the exact dimensions are arbitrary and can be adjusted according to the project's objectives. *See* **Images 9.1 and 9.2** taken from screen capture.



Images 9.1 and 9.2. Schematic rendering of the shoaling apparatus. The testing arena is a 13×18×29 cm filled with 5,000 mL dechlorinated tank water. The tank is divided into four equally sized quadrants. Image 9.1 shows fish dispersed throughout quadrants I, II, and IV. Image 9.2 shows fish more tightly grouped together in quadrant IV.

Drug Exposure-Induced Behavioral Changes/Circling Behavior Paradigm requires at least one 250 mL beaker for exposure, and a 6.5 × 13 cm Pyrex dish filled with 550 mL dechlorinated water. The exposure environment consists of 200 mL of water, including the diluted compound for the subjects to be exposed to. Consider that using more or less than 200 mL will change the amount of drug needed for dilution. The Pyrex testing environment consists of 550 mL pure dechlorinated water. This dish is large enough to show swimming behavior, yet small enough for the fish to exhibit the thigmotaxic/circling behavior.

3. Experimental Setup

3.1. Shoaling Paradigm

Suggested dimensions (although arbitrary) for the testing arena are 13×18× 29 cm, filled with 5,000 mL dechlorinated tank water. The bottom of the novel tank environment is divided into four equally sized quadrants according to the Cartesian system. When using a clear testing arena, the quadrants can be defined with a marker or tape on the rear of the arena, and still be sufficiently visible to be discerned from recordings by the researcher, without obscuring visibility of each individual fish.

The novel tank should be placed on a level, stable surface and sufficiently lit. Ideally, temperature (ambient and water) are kept as close as possible to the temperature fish experience in their home environment. Once transferred to the testing tank, the group of fish is recorded for 30 min.

3.1.1. Drug-Induced Behavioral Assessment

Individual zebrafish are transferred from their home tank to a 250 mL beaker filled with 200 mL dechlorinated water (control) or 200 mL dechlorinated drug treated water. Each subject is randomly assigned to a treatment group and immersed in a solution containing the drug for 1 h. During this time, a camera placed perpendicular to the exposure beaker records the one-hour session. If multiple beakers are used simultaneously, the view to neighboring beakers is to be obstructed with a plain barrier. Beakers should be placed on a level, stable surface and ambient and water temperature should be kept as close to the home tank environment as possible.

3.2. Circling/ Thigmoxactic Behavioral Assessment

Immediately after drug exposure, each fish is transferred to a circular 6.5×13 cm Pyrex dish filled with 550 mL dechlorinated tank water. An aerial mounted camera records behavior during a 37-min session, but only 30-min are subsequently scored (the first 5-min are not scored because the fish acclimate to the test environment and the last 2 min are not scored to ensure that behavior

was not influenced by the experimenter ending the session). The Pyrex dish should be placed on a level, stable surface. Ambient and water temperatures should be kept as close as possible to the temperature experienced in the home tank environment. Placing the Pyrex dish on a slide warmer is a good way to maintain home tank temperature.

4. Behavioral Endpoints

During the shoaling paradigm, observers can score the frequency of the fish entering each quadrant. Fish located in the same quadrant are in closer proximity to each other and are considered to be displaying shoaling behavior.

For the drug-induced behavioral changes measured during exposure in a beaker, an observer can score for immobility time, erratic swimming, and top time. A deviation in these behaviors from control fish can suggest a drug-induced behavioral change. Detailed definitions of each of these endpoints are outlined in **Table 9.1**.

For the circling behavior paradigm, an observer only scores the number of complete (360°) laps an individual fish performs around the circular testing environment. After behavioral data are collected and analyzed, comparisons can be made between experimental and control groups.

Table 9.1

Behavioral endpoint	Definition	Interpretation
Immobility	The time a subject spent without movement in any direction	Immobility or freezing can be indicative of anxiety and fear
Erratic swimming	Swimming in an irregular and jostling fashion, darting motions and rapid looping movements	Erratic swimming is an indicator of fear/anxiety. The frequency of erratic swim patterns increases in zebrafish exposed to stressors
Top time	Top time is measured as the time spent in the top half of the exposure beaker	Increased time spent in the top of the exposure beaker could be indicative of a need for an oxygen environment. It is also thought to be indicative of decreased anxiety
Circling behaviour	Defined as the number of times a fish makes a complete 360° lap around the circular testing arena (Pyrex dish)	A significant increase in circling behavior is indicative of hyperactivity and thigmotactic display

5. Time Requirement

The time required for the protocol will depend on the number of groups to be tested in the shoaling paradigm, and the number of individual fish to be tested for thigmotaxic display. Shoaling behavior can be assessed in 30 min per group, and thigmotactic behavior is assessed for 1 h per fish. As such, a typical experiment can span from 1 to 2 weeks to collect all behavioral data. Analysis can be performed over the course of a week, depending on the number of groups tested.

6. Data Analysis

6.1. Shoaling Behavior

The Cartesian system is utilized in this protocol, and the distribution frequency of fish is calculated over a 30-min trial for each of the four quadrants. Each quadrant (Q) is to be labeled using the Cartesian coordinate system in a counterclockwise fashion (top right Q I, top left Q2, bottom left Q3, and bottom right Q4).

Each 30-min session is divided up in 5-min time blocks for analysis (0–5, 5–10, 10–15, 15–20, 20–25, 25–30 min) to demonstrate change in schooling habits during drug exposure as a function of time. For each time block, the average frequency of fish (tabulated every 10 s) can be calculated per quadrant.

Nonparametric analyses can be applied first to evaluate the distribution of fish at a particular time point compared to a known probability distribution. The frequencies can be analyzed with the exact multinomial test to compare to known frequency distributions.

Secondly, multinomial tests can be deployed at every 10-s interval for each 5-min time block to account for variations in independent measures. An average p-value (cumulative probability) can then be computed and graphed to illustrate significant shoaling on the part of the treatment and control groups.

6.2. Drug-Induced Behavioral Changes/Thigmotactic Analysis

Each subject is randomly assigned to a treatment group and immersed in drug-treated, dechlorinated water. During the 60-min immersion time, all behavior is videotaped with a perpendicularly placed video camera. Behavioral output can be scored by observers blind to treatment conditions. Scoring can be done at set time intervals for a duration of 30 s. Time intervals at which to score behavior are recommended to be 5, 10, 20, 30, 45, and 55 min, based on increments outlined by Swain and colleagues

(2004). Observers can score for immobility, erratic swimming, and top time (*see* **Table 9.1** for details).

Following one-hour exposure at one of the aforementioned treatment conditions, subjects are placed in a Pyrex dish of dechlorinated tank water for 37 min while their behavior is recorded. The behavioral output is subsequently scored by observers blind to treatment conditions. Observers score based on the number of complete laps (swimming 360° around the dish) completed in 30 s at five different time points (5, 10, 15, 20, 25 min).

Statistical analysis can be performed to investigate the different effects of a drug or dosages versus a control group on the number of laps (i.e., thigmotactic behavior) displayed by the fish.

7. General Procedure

7.1. Shoaling Behavior

Groups of ten zebrafish are housed together in communal home tanks, and randomly assigned to a treatment group (drug dose 1, drug dose 2, etc. vs. control). Groups of ten fish are transported from their home tank either directly to the experimental environment or to a pre-exposure tank. If the experiment calls for pre-exposure to a drug or compound, the suggested pre-exposure time is one hour in a separate tank that is sufficiently aerated. Any drug or compound should be fully dissolved or mixed in sufficient dechlorinated tank water to hold the complete group (ten subjects) for the duration of the pre-exposure time. Subsequently, fish can be transported in a net from the pre-exposure tank to the testing arena. The testing environment can consist of either 100% dechlorinated tank water or can be spiked with an identical drug concentration as used in the pre-exposure period. In the arena dimensions suggested in this protocol, the arena would then consist of either 5,000 mL of dechlorinated tank water (for control groups) or a drug dose. For example, when assessing shoaling behavior during ethanol exposure, zebrafish can be transferred directly to the testing environment consisting of dechlorinated tank water treated with 0.25, 0.50, or 1.00% v/v EtOH. After the 30-min trial is completed, the group of fish can be transferred back to their home tank for further future testing or tissue analysis.

7.1.1. Drug-Induced Behavioral Changes

Individual fish are arbitrarily assigned to a treatment group, while ensuring male and female ratios among all treatment groups are balanced. Depending on the project requirements, at least one group will consist of the drug-treated individuals, and an equivalent number of fish will make up a control group. If different

dosages are to be tested, then several drug-treated groups can be formed to investigate dose-dependent behavioral effects.

Each fish is transported from the home tank to a 250 mL beaker containing either dechlorinated water or drug-treated dechlorinated water. Fish remain in the exposure beaker for 60 min while the sessions are videotaped. After the 60-min exposure period, fish can be transported from the beaker into a Pyrex dish containing fresh dechlorinated water and recorded for 37 min with an aerial mounted camera. After the session is completed, fish are transported back into their home tank or sacrificed for tissue analysis.

8. Anticipated/ Typical Results

This protocol was utilized to test the effects of ethanol, the dopamine receptor agonist SKF 38393, and the glutamate (NMDA) receptor antagonist MK-801 (*see* **Figs. 9.1 and 9.2**). These compounds were chosen for their observed effects on both rat and human performance. Previous reports have shown that these compounds are also behaviorally active in zebrafish (3, 14–16)

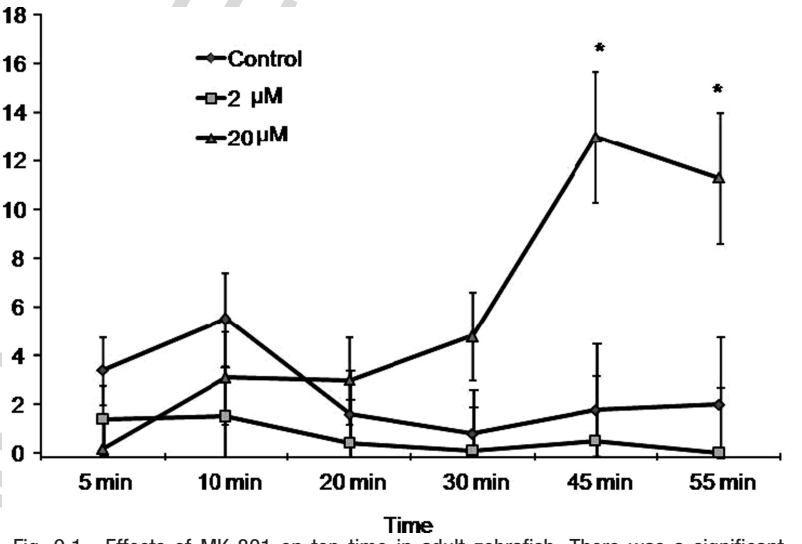


Fig. 9.1. Effects of MK-801 on top time in adult zebrafish. There was a significant effect of session time and a significant interaction effect during acute exposure to the NMDA antagonist. As the session progressed, zebrafish increased the amount of time spent in the top half of the exposure beaker at the highest dose (20 μM; $p<0.05$). Mean differences were analyzed using a mixed model ANOVA and Fisher's LSD post-hoc tests were used to indicate direction of effects where significant. Mean (±SEM) time (seconds) for each condition is shown, $^{*}p<0.05$.

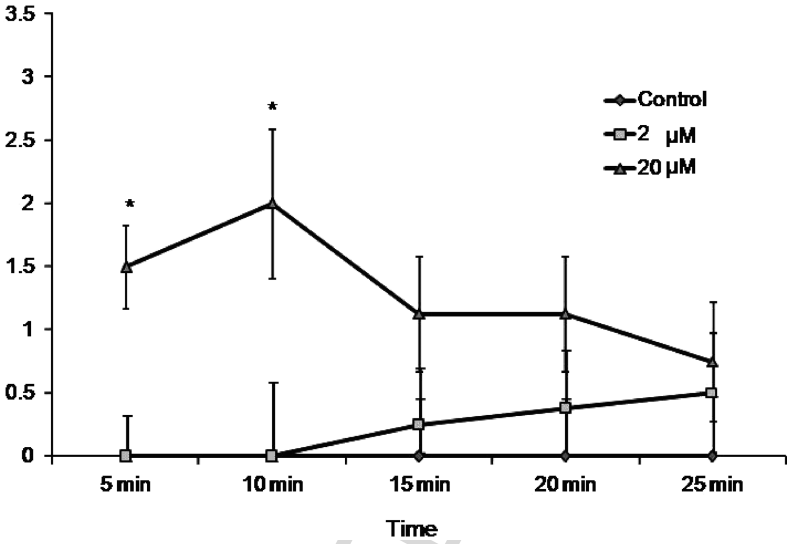


Fig. 9.2. Effects of circling behavior following acute treatment with MK-801. The effects of MK-801 on circling behavior show the largest dose (20 μM) significantly increased circling behavior over the 2 μM dose and controls ($p<0.05$; $n=24$). Mean differences were analyzed using a mixed model ANOVA and Fisher's LSD post-hoc tests were used to indicate direction of effects where significant. Mean (± SEM) circles completed during 30 s for each time point for each dose are shown.

The use of the experiments outlined in this protocol can be deployed to observe the gross behavioral effects of a novel compound on zebrafish. This can be useful in determining a dose range to be used in more elaborate testing procedures.

8.1. Shoaling Behavior

Shoaling is a highly developed and species-specific phenomenon in zebrafish. This display of social behavior can be disrupted with drugs, and offers method of detecting relatively mild changes in behavior. Using this protocol and exposing zebrafish to either MK-801, ethanol, or SKF 38393 yielded significant alterations in shoaling patterns for two of the three drugs under investigation (1.0% ethanol did not disrupt shoaling on this task). Zebrafish were divided into groups and tested in either pure dechlorinated tank water (control) or dechlorinated infused with either one of the abovementioned drugs. **Figures9.3A, B** and **9.4** show typical results for such an experiment. In summary, fish exposed to a MK-801-treated arena showed significantly different shoal distribution over time as compared to control groups. The distribution (average frequency) of fish was, in the first time block (0–5 min), found to be briefly significantly grouped ($p=0.03$). The remaining distributions for the session were not significantly different from chance (p's>0.05). **Figure 9.4** illustrates the average exact probability (p-value) for each time block during MK-801 treatment, which largely agrees with quadrant analyses.

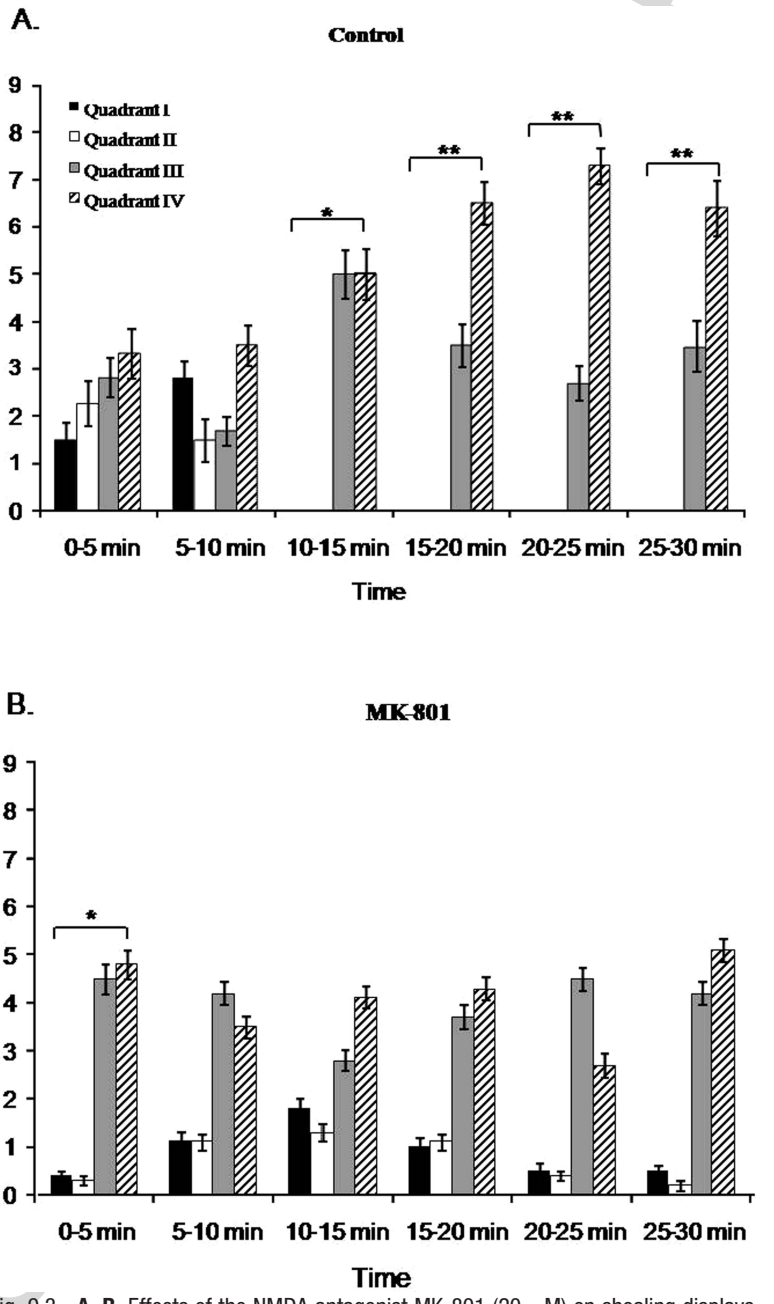


Fig. 9.3. **A, B.** Effects of the NMDA antagonist MK-801 (20 μM) on shoaling displays. (**a**) The control group (n=10) initially shows a lack of shoaling behavior before becoming acclimated to their surroundings; fish began shoaling around 10 min into the session as shown by significant multinomial probabilities across the remaining time blocks (p's<0.05). (**b**) Fish exposed to MK-801 (n=10) exhibited greater disruption of shoaling behavior than controls. Shoaling behavior was briefly displayed (p=0.03), but as the session progressed, fish began to display disorganization that proceeded until the end of testing. Multinomial statistical analyses were used to calculate p-values from mean frequencies of each quadrant. Mean (±SEM) frequencies are shown, *p<0.05; **p<0.001.

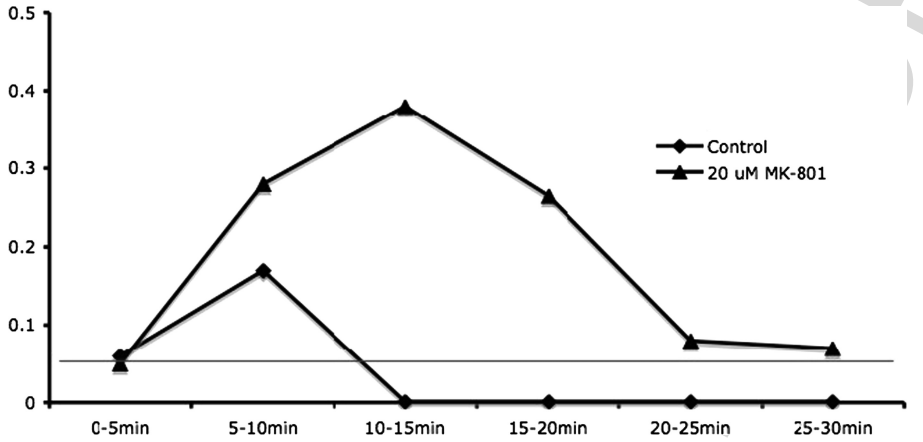


Fig. 9.4. Distribution of cumulative probabilities reflecting the effects of MK-801 (20 μM) on shoaling behavior. Data points represent average *p*-values of each time block for control and drug groups. Interpretation of data indicates a dichotomous relationship with respect to significance. Cumulative probabilities show similar trends to quadrant analyses. The first 10 min reveals nonsignificant *p*-values for control fish after which they become and remain significant throughout the session. Fish exposed to MK-801 briefly shoal (p=0.03; 0–5 min) the group, then becomes disrupted and disorganized for the remainder of the session (p's>0.05). Multinomial tests were used to calculate exact probabilities at 10 s intervals during open-field testing. Significance is indicated by the dashed line labeled at 0.05.

The results of exposure to SKF 38393 (100 μM in 5,000 mL of tank water) also indicated a deviation in shoaling distribution over time as compared to controls. **Figure 9.5A, B** illustrates the results of multinomial analysis of quadrant frequency during SKF 38393 exposure. Similar to control fish during MK-801 testing, subjects not exposed to the dopamine agonist showed an initial (0–5 min. time block) acclimation period for 5 min followed by shoaling display for the remainder of the session. Multinomial tests were significant for each quadrant for the remainder of the session (p's<0.05). Exposure to SKF 38393 initially mirrored that of controls with a nonsignificant *p*-value during the first 5 min (p=0.86) followed by brief shoaling in the 5–10 min time block (p<0.05). Results of statistical analyses revealed non-significant *p*-values for the remaining time blocks in the session: cessation of shoaling (p's>0.10). A graphical representation of the cumulative probabilities for SKF 38393 can be seen in **Fig. 9.6**. Again, fish exposed to the dopamine agonist initially follow the same results as control fish until the 10–15 min time block where the average multinomial probability becomes nonsignificant and shoaling behavior is disrupted.

8.1.1. Drug-Induced Behavioral Changes

The analysis of drug-induced behavioral changes, using 250 mL beakers, can yield results indicative of dose ranges that cause significant behavioral changes, or lethality, which can then be utilized for more fine-tuned behavioral testing. Using this protocol, the strongest effect was seen with the NMDA antagonist

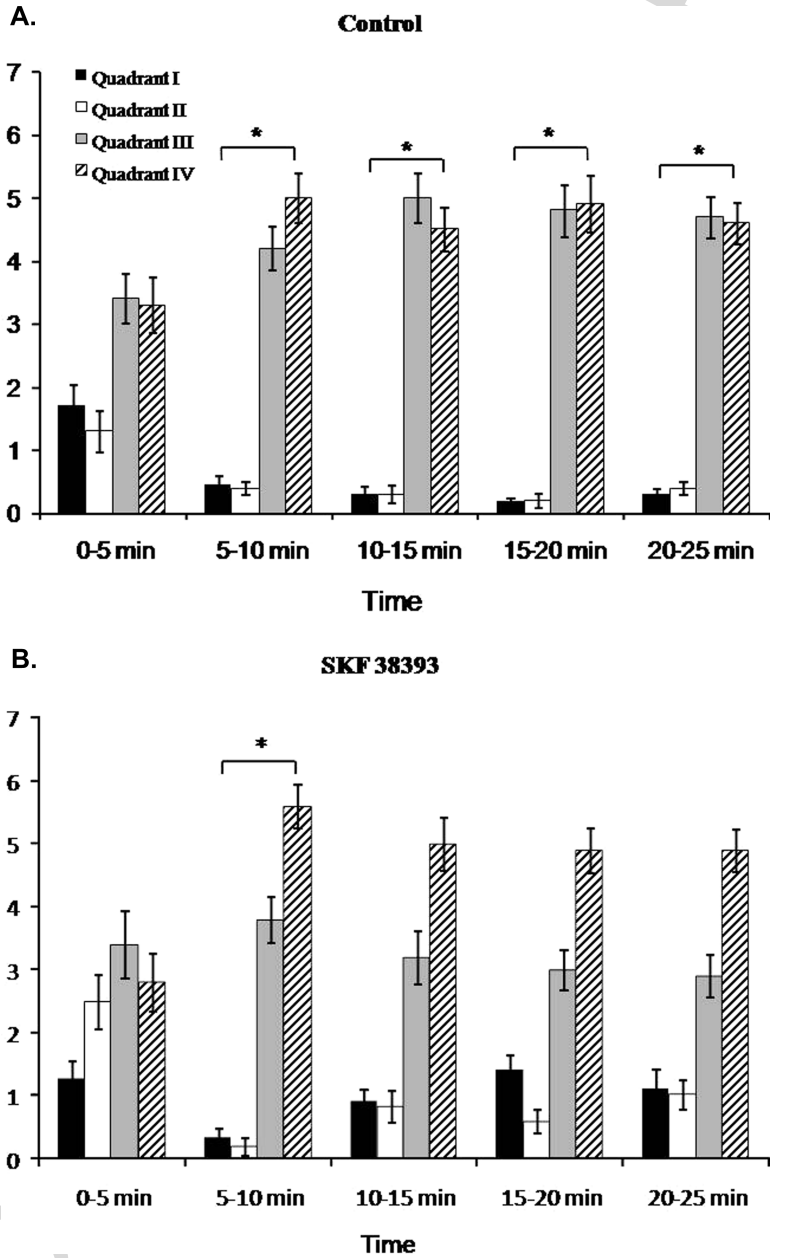


Fig. 9.5. **A, B.** Effects of the dopamine D1 subtype agonist SKF 38393 (100 μM) on shoaling displays. (**a**) The control group (n=10) after 5 min of acclimation display shoaling for the remainder of the session as shown by significant multinomial probabilities across the remaining time blocks (p's<0.05). (**b**) Fish in the SKF 38393 group followed the same trend as the control group in which probabilities were not significant for the first 5 min (p=0.86) and significant during the 5–10 min time block (p=0.03). The remainder of the session witnessed a disruption in shoaling behavior with nonsignificant multinomial probabilities (p's>0.05). Multinomial statistical analyses were used to calculate p-values from mean frequencies of each quadrant. Mean (±SEM) frequencies are shown, *p<0.05.

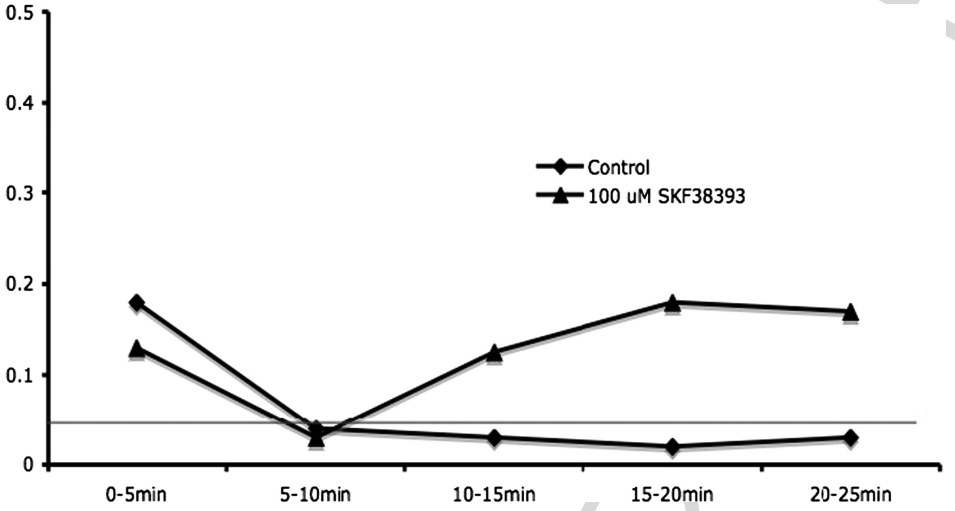


Fig. 9.6. Distribution of cumulative probabilities reflecting the effects of the dopamine agonist SKF 38393 (100 μM) on shoaling behavior. Data points represent average *p*-values of each time block for control and drug groups. Multinomial tests were used to calculate exact probabilities at 10 s intervals during open-field testing. Significance is indicated by the dashed line labeled at 0.05.

MK-801. Exposure to this drug resulted in increased time spent by each subject closer to the water surface. There was a significant effect of time [$F(2.52,62.93) = 3.21$, $p<0.05$] and a significant interaction [$F(5.03,62.93) = 5.09$, $p<0.01$] in the highest dose group (20 μM) for top time behavior. **Figure 9.1** illustrates typical effects of a drug (in this case MK-801) on top time during an acute 1-h exposure. Post hoc testing (Fisher's LSD) revealed that this protocol offers a reliable method to test drug-induced behavioral differences among treatment groups.

8.2. Circling Behavioral Analysis

When analyzing post-drug exposure thigmotactic behavior (circling) in a Pyrex dish, zebrafish are exposed to just dechlorinated tank water. The testing environment is not spiked with the drug or compound the fish were previously exposed to, so it is feasible that a change in behavior over time, as the drug effects wear off, is observed. An abnormal increase or decrease in circling behavior can be attributed to drug effects and has shown to be a reliable measure of gross drug-induced behavioral changes. Using MK-801 during pre-testing exposure yielded a significant increase in circling behavior when compared to control [$F(2,21) = 3.77$, $p<0.05$]. This protocol is particularly well suited to investigate the effects of drugs on thigmotactic display, and thus might not always yield significant results depending on the compound used. For example, although MK-801 showed a significant increase in circling behavior, SKF 38393 and ethanol did not. Typical results that can be expected in this paradigm are summarized in **Fig. 9.2**.

9. Trouble shooting

If, in the shoaling paradigm, a fish is found to be isolated against a quadrant marking or on the border, then the head of the fish is counted as the midpoint of the body axis and the corresponding quadrant is recorded. This is done to represent the swim path of the fish, and thus include the fish into the quadrant toward which it was traveling. During the shoaling paradigm, observers score the frequency of the fish entering each quadrant. Fish located in the same quadrant are in closer proximity to each other and are considered to be displaying shoaling behavior. One could imagine a scenario where two fish could be 2 cm apart in quadrant 1, but two other fish could be 1 cm apart in two different quadrants. Keep in mind that this task assess the overall group behavior for ten fish (using multiple sampling), so this potential influence of this scenario is negligible. It is also important to consider that thigmotaxic behaviors (as measured in the Pyrex dish) can be influenced by extraneous variables. Care must be taken to minimize vibrations to the apparatus caused by experimenter movement or the use of laboratory equipment. When considering both paradigms efforts should be made to minimize shadows that would project on to either test field, as this can simulate the presence of a predator and drastically alter behavior. Effort should also be put forth to ensure that the water temperature remains consistent between home and testing environments (drastic changes in water temperature can adversely influence behavior). Lastly, although we did not employ a video tracking device, such a device could prove to be quite useful for data collection. We did assess inter-rater reliability between any two raters manually scoring behavior. Typically, the percent agreement between any two raters was between 94 and 98%.

10. Conclusion

Behavioral paradigms can provide great insight into cognitive processes. The use of behavioral testing can enrich our understanding of drug-induced changes in the brain and in behavior (17). In zebrafish, shoaling is a highly developed aspect of this species' social repertoire, and can be deployed to investigate drugs that may have a neurological effect. Particularly, this paradigm has proven useful when utilizing drugs to manipulate NMDA and dopamine receptors (18). This protocol aims to facilitate behavioral research with a relatively simple and robust behavioral

paradigm. As investigating drug-induced effects often call for a large sample size, it is paramount to develop behavioral paradigms that are simple, fast, robust, and suitable for high-throughput screening. Zebrafish are inherently suited for high-throughput studies (19), and this protocol offers a set of experiments that are easy to conduct and lend themselves well for the analysis of a large number of subjects in a relatively short time frame. From this, future direction could move toward assessing individual zebrafish pairs. This would help us to better understand the hierarchy of behaviors and the complexity of the inter-relationship between conspecifics that ultimately result in the group dynamics related to shoaling.

Acknowledgments

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Chapter 9

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Chapter 10

Measuring Agonistic Behavior in Zebrafish

Henning Schneider

Abstract

Adult zebrafish (*Danio rerio*) are territorial, show aggressive behavior, and establish dominant-subordinate hierarchies. Here, a protocol for a standard opponent setup is described, which enables the identification, characterization, and quantification of agonistic activities of zebrafish. Following a period of social isolation, zebrafish are placed into an observation tank, engage in an agonistic encounter, and establish a dominance hierarchy within 15 min. The analysis of the behavior includes quantification of activities such as lateral display, frontal display, chasing and nipping over the course of the observational period with the help of The Observer® program. As a result, an event plot and a histogram are generated, which show the dynamics of agonistic activities. The described method can be used to quantify the effects of drug treatment or to identify modified activity patterns in mutant zebrafish.

Key words: Aggression, social isolation, dominance, subordinance, event plot, histogram.

1. Introduction

Agonistic behavior is characterized by a repertoire of diverse, complex, and species-specific activities that are displayed in encounters, which lead to dominance hierarchies (1, 2). Aggressive activities such as biting may be part of agonistic behavior, but establishing hierarchies is not dependent on the display of aggressive activities (3). The identification and quantification of individual agonistic activities are necessary for the understanding of their associations within a behavioral matrix (4). Ideally, changes in patterns of agonistic activities can be detected in genetically modified animals and can be linked to genes.

Fishes represent some of the best animal models for studies of social and agonistic behavior, because they can be observed

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easily in an artificial environment in the laboratory or in the field (5–9, 3). Underlying neurobiological and genetic mechanisms that have been identified in fishes such as cichlids and trout also play an important role in the regulation of agonistic behavior in mammals and humans (10–13). Zebrafish (*Danio rerio*) have emerged as one of the best animal models for the identification of genes, which play an important role in the regulation of behavior (14–16). Recent developments in molecular techniques such as gene trapping, gene knockdown, and gene knockouts provide powerful tools that will facilitate the behavioral genetics of zebrafish and other fishes (17–19).

The approaches to study agonistic behavior in fishes are diverse and are based on the characteristic activities of individual species. Siamese fighting fishes are among the group of fishes that have been studied extensively. An individual fish responds to its mirror image with a thread display during which the opercula are flared (20). Models or dummies of con-specifics have been used to trigger thread display (21). In addition, when two Siamese fighting fish are placed into an observation tank (standard opponent set-up), the activities that can be recorded and quantified include bites (open mouth contact), aggressive display during which the fins are extended, and aggressive display with opercula fully erected (22).

Agonistic behavior in cichlids has been measured under controlled conditions in an observation tank in which two individuals have been housed in separate compartments and were allowed to establish a territory over a period of several days (3). Territorial behavior is videotaped until one animal has lost the fight and swims away or is being chased by the winner animal. Similar to Siamese fighting fish, studies in cichlids have employed mirrors, models, and pairing with conspecifics (3). Moreover, the change of the coloration such as the black eye bar in cichlids is used to distinguish territorial from nonterritorial animals (6, 7).

Schooling fish like the Atlantic salmon, trout, or zebrafish also establish dominance hierarchies and engage in agonistic encounters as they compete for mating partners and food. In juvenile Atlantic Salmon, territorial behavior has been described as a pattern of agonistic activities that include charging, nipping, chasing, frontal display, lateral display, and fleeing (8). Trout show similar agonistic activity within a group (23) or in an intruder test (24). Zebrafish agonistic behavior has been studied in different behavioral contexts using different experimental designs (25–27). In the context of population density and sex ratio, aggressive behavior has been defined by two behavioral elements: repel and circle (27). In a different study that uses the standard opponent setup, aggressive behavior was measured by counting chases and bites (26). Moreover, aggression in zebrafish has also

been approached by measuring the response of individuals to mirrors and computer-animated animals (28, 25, 29, 30).

Here, we describe our standard opponent setup for measuring agonistic behavior that leads to dominant-subordinate hierarchies in wild-type zebrafish. By analyzing standard opponent encounters we identified several agonistic activities: lateral display, frontal display, chasing, and nipping (**Fig. 10.1**). During chasing a dominant animal approaches a subordinate animal from behind. Subordinate animals turn away and escape in order to avoid any physical contact with chasing animals. During a lateral display both zebrafish swim parallel to each other in opposite direction and circle with their dorsal fins raised and caudal fins extended. Animals avoid initial direct contact with each other during this activity. Nipping is often associated with chasing, includes physical contact and ends a chase. A frontal display is recorded when two fish approach each other from the front with the attempt to nip or bite. Before a hierarchy is established, we see also schooling behavior. Both fish swim together as a pair in the absence of any agonistic activity. The fish are close to each other but they do not make physical contact. The repertoire of agonistic activities is

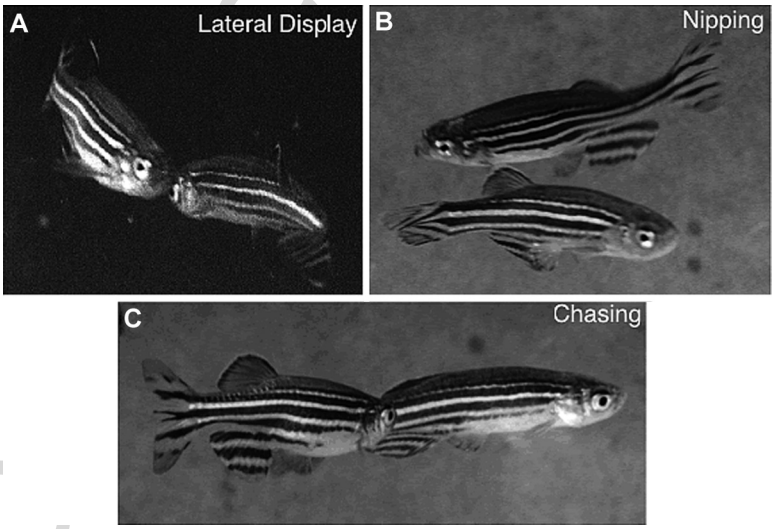

Fig. 10.1. The figure shows photographs of (**a**) lateral display, (**b**) nipping, and (**c**) chasing. During lateral display (**a**), both fish raise their fins, position themselves parallel to each other in the opposite orientation, and *circle* each other. The heads of both fish point toward the tail fin of their opponent. No physical contact occurs during lateral display. In contrast, nipping includes physical contact (**b**) shows the beginning of a nipping. The chasing fish (*) has turned toward the opponent (°) just before it attempts to nip the opponent mid-body. In contrast to the lateral display, the opponent is pointing its head away from the attacking fish and tries to swim away. An example of a chase is shown in (**c**). The dominant zebrafish (*left*) approaches the mid-body region of a subordinate zebrafish (*right*) from posterior. Note that the fins of the subordinate fish are lowered while the fins of the dominant fish are raised and expanded.

displayed reliably and repeatedly in different pairs so that comparisons of encounters of zebrafish with different genetic makeup are possible.

The analysis of agonistic encounters in the standard opponent setups as described here focuses on the overall pattern of agonistic activities of the pair. The timing of agonistic activities over the observational period provides information about the apparent stereotypic organization of this behavior in zebrafish. Screening of mutant zebrafish lines for changes in the activity pattern may lead to the identification of genes that participate in the regulation of agonistic behavior. Generating event plots of standard opponent encounters of different species of fishes may be helpful for exploring the evolution of agonistic behavior.

2. Materials and Methods

2.1. General Instructions

Animals used in the experiments should be maintained under identical environmental conditions. Light and dark cycles of 14 and 10 h, respectively, and a water temperature of 28°C are standard in zebrafish facilities (31). If a zebrafish facility is not available, fish can be kept in a large 20-gallon aquarium equipped with a heater, aquarium light on timer, and pump. If animals are obtained from local pet shops, we followed standard recommendations: quarantined and equilibrated animals for 7–14 days before they were used for behavioral experiments (31). The animals that have been selected for observation should be separated as individuals for at least two or more days before the actual observation and recording of encounters (for details *see* **Section 2.2**). This can be achieved by keeping animals in small opaque freezer boxes that are maintained in trays. Small openings in the freezer boxes will allow the exchange of water so that the water quality for each fish is identical. To generate naïve social animals individuals can be separated early in their larval life (32). Depending on the goal of the experiment, this can easily be done in zebrafish by raising individual zebrafish larvae in small containers such as modified, opaque 50 mL centrifuge tubes or scintillation vials with plastic mesh bottoms (31). We preferred to set up observational tanks in a dedicated observation room to avoid interference and distraction. Observations should be scheduled at the same time of the day since overall activity of zebrafish varies over the course of the day. We recorded encounters between 10 AM and 1 PM. Described methods have been approved by IACUC at DePauw University and William Paterson University.

2.2. Animals

Weight and size of individuals of an opponent pair should not differ more than 10%, since larger zebrafish are more likely to become dominant. We weighed 20–30 wild-type zebrafish (Ekkwil) to obtain about ten good matches. We have anesthetized the selected fish in Tricaine (0.01–0.02%) for about 5 min or until the fish stop swimming spontaneously. Then, they are transferred to a weighing dish or to a 50 mL beaker filled with 20 mL system water. The weight and sex of fish is recorded, and the fish are transferred to labeled containers (one fish per container). The sex of animals should be recorded, since male–male pairings show a slightly larger number of agonistic activities compared to male–female or female–female pairings. Since the time of social isolation affects the fighting activity, all fish used in a series of experiments should be kept in social isolation exactly and consistently for the same time period. Animals kept in social isolation for at least 2 days establish hierarchies within 30 min. Shorter social isolation can lead to less fighting activity and fish may not establish a hierarchy.

2.3. Observation Tank

We used a 2.5-gallon glass aquarium as observation tank. The back and sides of the observation tank were covered with aquarium background. The tank can be filled up to 0.5 inch with clean gravel. A small heater was used to maintain the water temperature in the observation tank at 28°C. A Plexiglas cover and a light source such as an aquarium light should be available. A bubbler was used to aerate the water when fish were not in the tank. An opaque divider was used to separate the right and left half of the tank.

2.4. Video Camera Setup

A standard Sony video camera with DV-R recording capabilities was set up in front of the tank and behind a barrier so that the fish cannot see the observer or operator of the camera. The red recording light of the camera should be taped over. Lights in the recording room were turned off to reduce reflections.

3. Procedure

1. Select two zebrafish of similar size and weight (within 10%) that have been kept in social isolation for exactly the same time. Record the sex of selected zebrafish and use the same pairing (male–male, female–female; female–male) throughout the series of experiments.
2. Place one fish into the left and right compartment of the divided tank. Make sure that there are no gaps through

which the fish can sneak into the other compartment. Remove the bubbler and cover the tank with the Plexiglas cover. Put the light source on top of the cover. Animals should equilibrate to the new environment for 30 min. The equilibration period can be expanded but identical equilibration periods should be used consistently for all experiments. The zebrafish should not be disturbed during the equilibration period.

3. Following the equilibration period turn on the camera. Gently lift up the Plexiglas cover with the light source on top and remove the divider. Removal of the divider should be done carefully so that no "dirt" is stirred up. Gently, place the cover with the light source back onto the tank and move away from the observation tank.
4. Continue the recording for 30 min. Wild-type fish establish a hierarchy within 15 min as indicated by the dominant zebrafish chasing the subordinate fish. Different strains of zebrafish or treatment of fish may affect the time at which a hierarchy is established. Turn off the camera at the end of the observation period.
5. Place the divider back into the observation tank to separate the animals.
6. If animals are used for a consecutive fight on the next day, they can be transferred to their individual containers. The animals should not be kept together for an extended period of time without a divider.

3.1. Analysis of Recorded Activities

A total of 10–12 encounters are used in an experimental series. For a quantitative analysis of the behavior, the entire episode of a fight is played back and watched on a TV monitor or computer screen. Individual activities are registered with the help of The Observer® program (Noldus, USA). Using The Observer®, the computer can be programmed so that each single activity such as lateral display, frontal display, chasing, or nipping has its own designated key on the keyboard (L for lateral display, N for nipping, C for chasing, F for frontal display). A single tap of a key will register one occurrence of an agonistic activity between the two animals. The advantage of this procedure is that the quantification of activities and a timeline of events can be generated quickly. Our analysis has focused on the occurrence and pattern of agonistic activities of the pair and not on the behavior of each individual fishes. When individual activities occur very fast or are not clearly distinguishable, the recording can be played back at slower speed. Clear definitions of activities should be established before the analysis. For example, a lateral display ends when an opponent turns its head away from the other fish or changes

swimming direction. Chases are defined along the same lines: a chase begins when one fish (chasing fish) accelerates swimming speed while it approaches the opponent from posterior and ends when the chasing fish stops or slows down.

The Observer® program allows for a quick analysis of agonistic behavior that includes an event graph and a quantification of agonistic activities. An event plot shows the occurrence of single agonistic activities over the duration of the observational period and is used to characterize the length of different phases of the encounter (**Fig. 10.2**). A typical encounter starts with a phase of intense lateral display. Next, the fish engage mostly in nipping and frontal displays. In the final phase of an encounter, mostly chasing behavior is observed. At the time point, when the fish switch

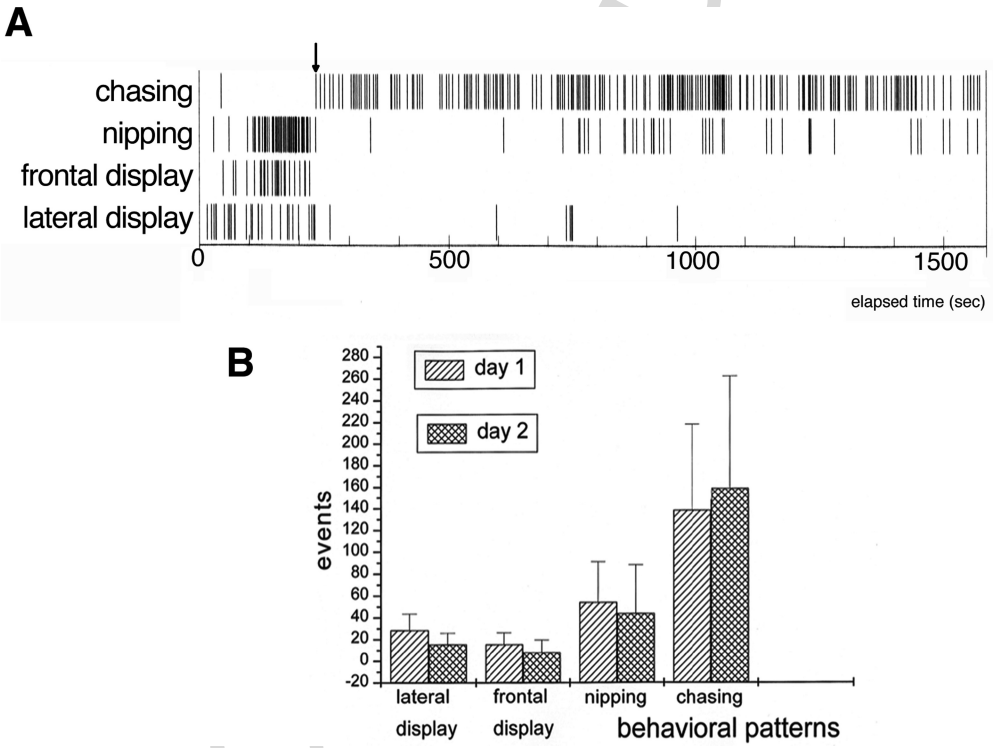

Fig. 10.2. (**a**) Shows an event plot of a typical single encounter of a pair of wild-type zebrafish. Four activities were recorded: lateral display, frontal display, nipping, and chasing. Each bar represents a single occurrence of a single agonistic activity of the pair. We did not discriminate between the two fishes. The duration of the observation period in this example was 1,600 s (26.6 min). Lateral displays occur after the divider has been removed. A period of physical interaction that includes nipping and frontal display follows. The *arrow* indicates the time (3 min 52 s) at which a dominance hierarchy was established. From this time on, the dominant fish chases the subordinate. Some chases end in nipping. Occasionally, a lateral display has been observed during the chasing phase of the encounter. (**b**) The histogram shows the average number of agonistic activities of the same six pairs (n=6) that were tested on two consecutive days shows: 28 (sd=15) lateral displays on day1, 15 (sd=10) on day 2; 15 (sd=11) frontal displays on day1, 8 (sd=8) on day 2; 53 (sd=39) nippings on day 1, 42 (sd=46.5) on day 2; 139 (sd=82.5) chases on day 1, 158 (sd=110) on day 2. There is no significant difference of agonistic activities on day 1 and day 2.

to chasing, the hierarchy has been established and the dominant animal chases the subordinate. Since both fish are kept in a small tank with no place to hide, the chasing continues to the end of the observational period. The quantification of encounters includes the number and frequency of agonistic activities and can be used for generating histograms. Overall, we counted 25 lateral displays, 11 frontal displays, 63 nippings, and 242 chases in the encounter shown in **Fig. 10.2a**. Lateral displays, frontal displays, and nippings seem to be important for establishing a hierarchy since these agonistic activities occur mostly before a hierarchy has been established. Since agonistic encounters show variations in onset and the number of activities, calculating the average number, frequency, and standard deviations of individual activities such as lateral display, frontal display, nipping, and chasing is performed. The histogram (**Fig. 10.2b**) shows the analysis of encounters of six pairs that were conducted on two consecutive days (30 min encounters on day1 and day2). The same pairs were observed on both days. The results show that fewer lateral and frontal displays occur than nipping and chasing. There is no significant difference between encounters on day 1 and day 2.

The data analysis as presented above does not include the activity of individual fish, since our focus has been on the overall pattern of agonistic activities of the pair and not the activity of individual fish, but can be expanded to record the activities of individual fish in an encounter. This would require tagging of individuals or labeling them electronically during video analysis.

4. Conclusion

The zebrafish represents an animal model with a stereotypic agonistic behavior, which can easily be studied in the laboratory. While we have studied the overall pattern of agonistic activities of a pair in a standard opponent setup, the experimental design and analysis can be used to explore the role of variables such as sex, age, and drug treatment on individual agonistic activities in individuals. Moreover, genetic tools that are available for zebrafish make it an excellent system to find genes that participate in the control of agonistic behavior in vertebrates.

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Chapter 11

Measuring Endocrine (Cortisol) Responses of Zebrafish to Stress

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Abstract

The zebrafish (*Danio rerio*) is rapidly becoming a popular model species in stress and neuroscience research. Their behavior, robustly affected by environmental and pharmacological manipulations, can be paralleled by physiological (endocrine) analysis. Zebrafish have a hypothalamic-pituitary-interrenal (HPI) axis, which is homologous to the human hypothalamic-pituitary-adrenal (HPA) axis. While mice and rats use corticosterone as their main stress hormone, both humans and zebrafish utilize cortisol. This protocol explains the whole-body cortisol extraction procedure and the use of the human salivary cortisol ELISA kit to measure the amount of cortisol in each zebrafish sample. The ability to correlate physiological data from individual fish with behavioral data provides researchers with a valuable tool for investigating stress and anxiety, and contributes to the utility of zebrafish neurobehavioral models of stress.

Key words: Zebrafish, physiological endpoint, HPA axis, HPI axis, stress, ELISA, cortisol.

1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis mediates the endocrine response to stress in humans and animals (1). Under stress, the paraventricular nucleus of the hypothalamus produces corticotropin-releasing factor (CRF), which is delivered to the anterior pituitary gland via the hypothalamic-hypophysial portal blood vessel system (2). CRF stimulates the anterior

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pituitary gland, causing the release of andrenocorticotropic hormone (ACTH) into the blood stream (3). When stimulated by ACTH, the adrenal cortex synthesizes glucocorticoid hormones from a cholesterol precursor (4, 5). Increased levels of glucocorticoids initiate metabolic effects that modulate the stress reaction (4, 6). These effects include the stimulation of gluconeogenesis, anti-inflammatory effects, and immune system suppression (7). The effects of the stress reaction are harmful in excess and are alleviated through a negative feedback to the hypothalamus and pituitary, which suppresses CRF and ACTH release (8, 3).

Mice and rats have traditionally been used for stress neuroendocrine research (9). Although they are phylogenetically closer to humans, rodent endocrine HPA systems utilize corticosterone as the main stress hormone (10). A similar mechanism has been found in teleosts, specifically zebrafish (*Danio rerio*) (11), whose hypothalamus-pituitary-interrenal (HPI) axis is homologous to HPA (**Fig. 11.1**). With cortisol being the main mediator of physiological response to stress, this makes zebrafish an excellent model for endocrine research (12–16). Here we report a simple protocol for analysis of whole-body zebrafish cortisol concentration as a physiological (endocrine) marker of stress and anxiety.

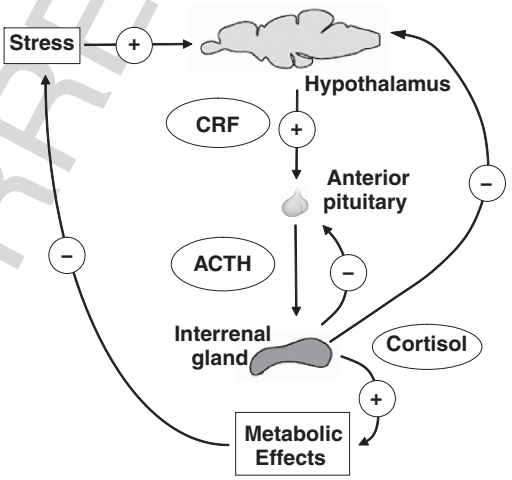

Fig. 11.1. The hypothalamic-pituitary-interrenal (HPI) axis in zebrafish. The hypothalamus secretes corticotropin-releasing factor (CRF), which stimulates the pituitary to release adrenocorticotropic hormone (ACTH). The interrenal gland, when affected by ACTH, secretes cortisol, the primary stress hormone in zebrafish. A negative feedback system acts on the hypothalamus to ensure homeostatic regulation. The human HPA axis functions similarly; however, it contains an adrenal gland in place of the interrenal gland.

2. Protocol

2.1. Animals and Housing

Adult (3–5 month old) zebrafish (male and female) can be purchased from commercial vendors. An acclimation period of at least 10 days must be given upon arrival into the laboratory environment. The fish are separated into groups of 20–30 and housed in 40-L home tanks. Deionized water is used in each tank and treated with Prime Freshwater and Seawater Concentrated Conditioner (Seachem Laboratories, Inc., Madison, GA). The room and tank water are maintained at 25–27°C with 12-h cycles of illumination with ceiling mounted artificial fluorescent lighting. Food is provided in the form of Tetramin Tropical Flakes (Tetra, USA). After behavioral study, fish are euthanized using 500 mg/L Tricane (Sigma-Aldrich, USA), and whole-body samples are used for cortisol assay (see further).

Cortisol extraction: Procedure for performing cortisol extraction on the whole body samples was adapted from Alderman and Bernier (13) and modified as described in Egan et al. (14).

(a) Following behavioral study, whole body samples are collected and frozen at –20°C (or lower) for biological study to assess cortisol levels.

(b) Body samples are partially thawed, weighed, and then homogenized in 500 μL of ice-cold 1X phosphate-buffered saline (PBS) buffer. After recording weight (g), the whole body samples are dissected on ice into smaller parts for efficient homogenization. Homogenization can be performed using a Tissuemiser® from Fisher Scientific (USA). Note: Measuring the weight of the whole body sample prior to homogenization is necessary for determination of cortisol concentration following extraction and ELISA (see further).

(c) The homogenizing rotor blade is washed with an additional 500 μL of ice cold 1X PBS and collected in a 2 mL tube containing the homogenate.

(d) The homogenizing rotor blade and probe must be washed with ethanol (100%) and deionized H_2O in-between each sample. This is an important step to minimize cross-contamination of samples.

(e) Samples are kept on ice throughout this process and then transferred to labeled glass extract-O tubes.

(f) 5 mL of diethyl ether (Fisher Scientific, USA) is added to each sample.

(g) The samples are vortexed for 1 min and then centrifuged at 3,500 rpm for 5 min.

(h) Following centrifugation, the organic layer containing cortisol was removed from each sample and placed in a separate test tube. The process was repeated two (or three) times consistently throughout the experiment to ensure maximal cortisol extraction. The cortisol-containing layer (organic phase) is usually yellowish in color.

(i) Samples are kept overnight in the fume hood to allow for evaporation of ether. Other methods of drying the organic solvent could be used, such as the speed vacuum centrifuge equipped with a cryotrap, or the evaporation to dryness under nitrogen sparge.

(j) Ninety percent recovery was confirmed for this protocol using (8) [H]-testosterone as a tracer for evaluation.

Cortisol ELISA Assay:

(a) Cortisol is reconstituted in 1 mL of 1X PBS after ether evaporation and incubated overnight at 4°C.

(b) ELISA is performed per manufacturer's instructions to quantify cortisol concentrations using human salivary cortisol assay kit (Salimetrics LLC, USA).

(c) ELISA color or reaction intensity is measured in a VICTOR-WALLAC (Perkin Elmer, USA) plate reader with the manufacturer's software package.

(d) Whole body cortisol levels are quantified using a 4-parameter sigmoid curve minus curve fit based on absorbances of standardized concentrations versus those observed in the samples. Cortisol levels are normalized based on the weight of the whole body sample and reported as absolute circulating cortisol concentrations (ng/g body weight).

Troubleshooting:

(a) As circulating levels of cortisol fluctuate throughout the sleep/wake cycle, it is important to perform behavioral experiments and sacrifice all subjects at the same time of day.

(b) Due to the volume of the tissue being homogenized, sectioning the whole body into smaller pieces prior to homogenization reduces the chance of losing material or jamming the equipment.

(c) The homogenizer must be carefully washed in ethanol and deionized water after each sample. Failure to fully wash and rinse the homogenizing blade will result in cross-contamination of samples that will distort results.

(d) Using glassware instead of plastic Eppendorf tubes helps reduce the loss of cortisol from samples. Cortisol tends to

stick to the sides of plastic containers, and thus a percentage is lost upon each transfer.

(e) Body size may affect the accuracy of cortisol detection. Use zebrafish of similar sizes. Embryonic or abnormally small zebrafish may result in a cortisol concentration below the ELISA assay's sensitivity threshold, and thus should be avoided if possible. Since the minimum threshold concentration has not been determined, it may be required to combine multiple samples when using embryonic subjects.

(f) Using a radioactive tracer (e.g., tritium) can be useful in determining the amount of cortisol lost during the extraction procedure; this proportion can be used to correct the concentration of cortisol per gram of fish for a more accurate analysis.

(g) Because equipment in different laboratories vary, it is possible to adapt the amount of ether used and the number of extractions done. Usually, a 1:3 to 1:5 solute:solvent ratio is used. To obtain the highest yield, repeat the extraction procedure several times. However, the amount of ether used and the number of extractions performed must be standardized for all samples used in the study.

(h) If the homogenate becomes an emulsion after adding ether, more ether before centrifugation may help separate the homogenate. However, if additional ether is used for separation, the remaining samples must similarly be treated for standardization and consistency of data.

(i) Before performing the salivary cortisol ELISA, make sure to graph the plate layout and the position of each sample (to assist in locating the samples for future quantification).

(j) Always handle hazardous materials with care and according to Institutional and laboratory guidelines. Ether emits toxic fumes and thus must be handled and evaporated in a fume hood. Radioactive materials require proper attire and conduct.

(k) Laboratory temperature may affect the outcome of the extractions. To prevent confounding the results, be sure to keep the lab temperature standardized throughout this procedure.

(l) Samples can be stored for a long period (several months) at –80°C before cortisol extraction procedure.

(m) In order to prevent cross-contamination, pipette tips must be changed after use and equipment must be cleansed after contact per sample.

(n) After centrifuging, the organic (containing cortisol) layer is the top layer and hence is the layer to be collected and stored.

(o) To prevent confusion, label each test tube properly. Be aware that ether will wash away marker labels on glass tubes if spilled.

2.2. Typical Results

Figure 11.2 represents data collected after drug exposure and withdrawal experiments performed on zebrafish in our laboratory in 2009. The consistency of the results in respect to increased whole-body cortisol concentrations following introduction of stressful stimuli is in line with behavioral data gathered in these and previous studies (14). Zebrafish behavioral research frequently uses the novel tank paradigm, a test that exploits the instinctive anxiety-like behavior induced by exposure to a novel environment. Numerous studies reported that new environment as well as additional stressors (e.g., presence of predators, alarm pheromone or drug withdrawal syndrome), lead to specific behavioral phenotypes (representative of anxiety) including decreased exploration, increased freezing, and increased erratic (darting) movements. In **Fig. 11.2**, paralleling this anxiety-like behavior (behavioral data not shown), whole-body cortisol analysis of anxious drug withdrawal zebrafish predictably reveals significantly increased cortisol concentrations.

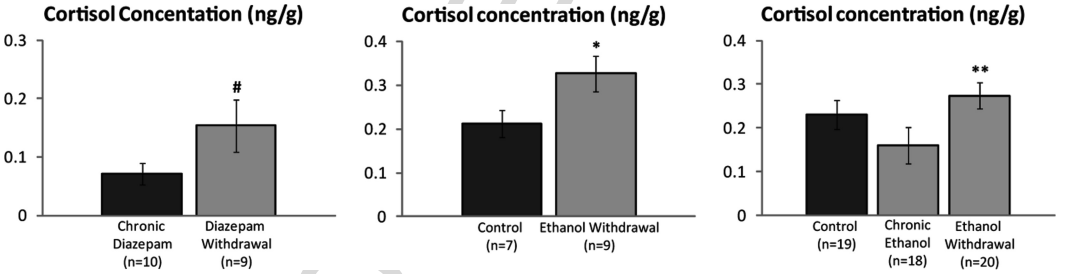

Fig. 11.2. Zebrafish endocrine responses (whole-body cortisol, ng/g fish) to withdrawal from diazepam and ethanol. *Left* to *right*: 72-h withdrawal from chronic diazepam (72 μg/ml, 2 weeks); 12-h withdrawal from chronic ethanol (0.3%, 1 week); chronic ethanol exposure (0.3%, 1 week) and 12-h withdrawal from chronic ethanol (0.3%, 1 week). Data are presented as mean ± SEM (*p<0.05, **p<0.01, #p=0.05–0.1, trend, U-test).

3. Discussion

The protocol described here represents a significant modification of previously employed cortisol assays for assessing physiological stress in zebrafish. In prior studies, human serum cortisol kits

necessitated combining multiple fish for a single cortisol sample. Here, we used human salivary cortisol kits that are sensitive to a full range of cortisol levels from 0.003 to 3.0 μg/dL. Such high sensitivity enables quantification of whole-body cortisol in individual fish samples. This methodological modification has significant consequences for the utility of zebrafish to study anxiety and stress disorders, because smaller sample groups can be used, and additional data becomes available to correlate behavioral and endocrine responses to stress in individual fish.

Analysis of the physiological (neuroendocrine) responses to stress in zebrafish is a valuable tool complementing behavioral studies. The cortisol extraction procedure and human salivary ELISA assay are relatively simple, inexpensive, and can be easily adopted in a variety of laboratory settings. Additional modifications to the protocol may enhance the yield further, as discussed in the troubleshooting section. Likewise, statistical analysis of correlation between behavior and endocrine response may further assist in data interpretation. For example, the Spearman's rank correlation coefficient, used to assess the relationship between two variables, can determine the level of correlation between behavioral data and cortisol concentration values.

Overall, the ability to parallel physiological responses (i.e., cortisol production) with behavioral responses provides researchers with an important tool for investigating stress-related responses. The use of human salivary cortisol assays provides physiological evidence measuring the endocrine stress response in individual zebrafish that can be associated with anxious behavioral responses. The method is also cost-effective, as compared to measuring cortisol levels using more expensive tools, such as mass spectrometry or gas chromatography. This new protocol offers a simple, fast, reliable, and cost-effective method to measure the endocrine stress response in zebrafish.

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Chapter 12

Phenotyping of Zebrafish Homebase Behaviors in Novelty-Based Tests

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Abstract

Various novelty-based assays used to quantify zebrafish (*Danio rerio*) behavior show a striking similarity to behavioral responses in rodents. Exposed to the open field test, zebrafish establish overt homebases demonstrating clear preferences for a particular area of the tank. This behavior aims to establish a "safe zone" that zebrafish can familiarize themselves with and feel secure in, and is similar to homebase behaviors of various laboratory rodent species. Here we outline a simple protocol for homebase phenotyping in zebrafish.

Key words: Zebrafish, homebase behavior, exploration, open field test, cognitive maps, spatial orientation.

1. Introduction

Animal exploratory behavior provides a robust source of quantifiable endpoints used in neuroscience and behavioral research (1, 2). Traditional exploration-based paradigms include the elevated plus maze (3), light–dark box (4), and the open field test (OFT) (5–7), extensively studied in rodents (6, 8–10).

The OFT paradigm has also provided important insights into animal motor and affective phenotypes (13, 14). Although the OFT has recently been applied to zebrafish (15–17), this research

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has primarily utilized larval animals (18). While larval zebrafish represent a popular and useful model in neuroscience research (19–21), they are not without some limitations. For example, larvae do not exhibit the rich behavior of their adult counterparts (21), and their behavior and cognitive abilities cannot be fully translated to adult subjects' behavior. Additionally, they lack fully developed neuromediatory and endocrine systems (22), as well as some neural circuits and projections (23). Our method, therefore, will focus on using adult zebrafish to characterize their neurophenotypes.

Homebase behavior is a naturally occurring phenomenon, as animals often select a home site to which they repeatedly return after exploring the surrounding territory (24). Perhaps even more importantly, laboratory rodents use these homebases as strategic "reference points" to orient and organize their exploration (24–26). For example, mice and rodents visit many places in a novel environment, but typically choose one or two zones to spend most of their time, also displaying the highest grooming and rearing activity (26).

Taken together, this emphasizes the fact that homebase formation represents an important aspect of animal exploration. Our observations suggest that homebase behavior exists in zebrafish, and may therefore play a role in the spatial organization of zebrafish locomotor behavior. Here we present the methodology to analyze and quantify this interesting behavioral phenotype in zebrafish also (*see* (27) paper for details).

2. Methods and Materials

2.1. Animals and Housing

Adult wild-type short-fin zebrafish (6–8 month-old; ≈50:50 male:female ratio) can be obtained from a local commercial distributor, and should be given at least 10 days to acclimate to the animal facility. Animals can be housed in groups of approximately 20–30 fish per 40-L tank. Tanks should be filled with deionized water, with room and water temperatures maintained at ≈25°C and water pH at 7.0–8.0. Illumination can be provided by ceiling-mounted fluorescent light tubes on a 12–12 or 10–14 h cycle, consistent with the zebrafish standard of care (29).

2.2. Apparatus

The zebrafish homebase paradigm can be established using several different novel OFT tanks. For example, in our experiments, OFT1 represented a large rectangular plastic opaque tank (12.3 height × 38.7 width × 47.3 cm length) divided into nine zones. OFT2 was a white plastic cylinder (23.6 height × 22.8 cm diameter) divided into nine zones, and OFT3 was a white square tank (14.0 height × 29.0 width × 37.0 cm length) with textured surface and rounded corners divided into eight sections (*see* **Figs. 12.1 and 12.2** for details). These three apparatuses

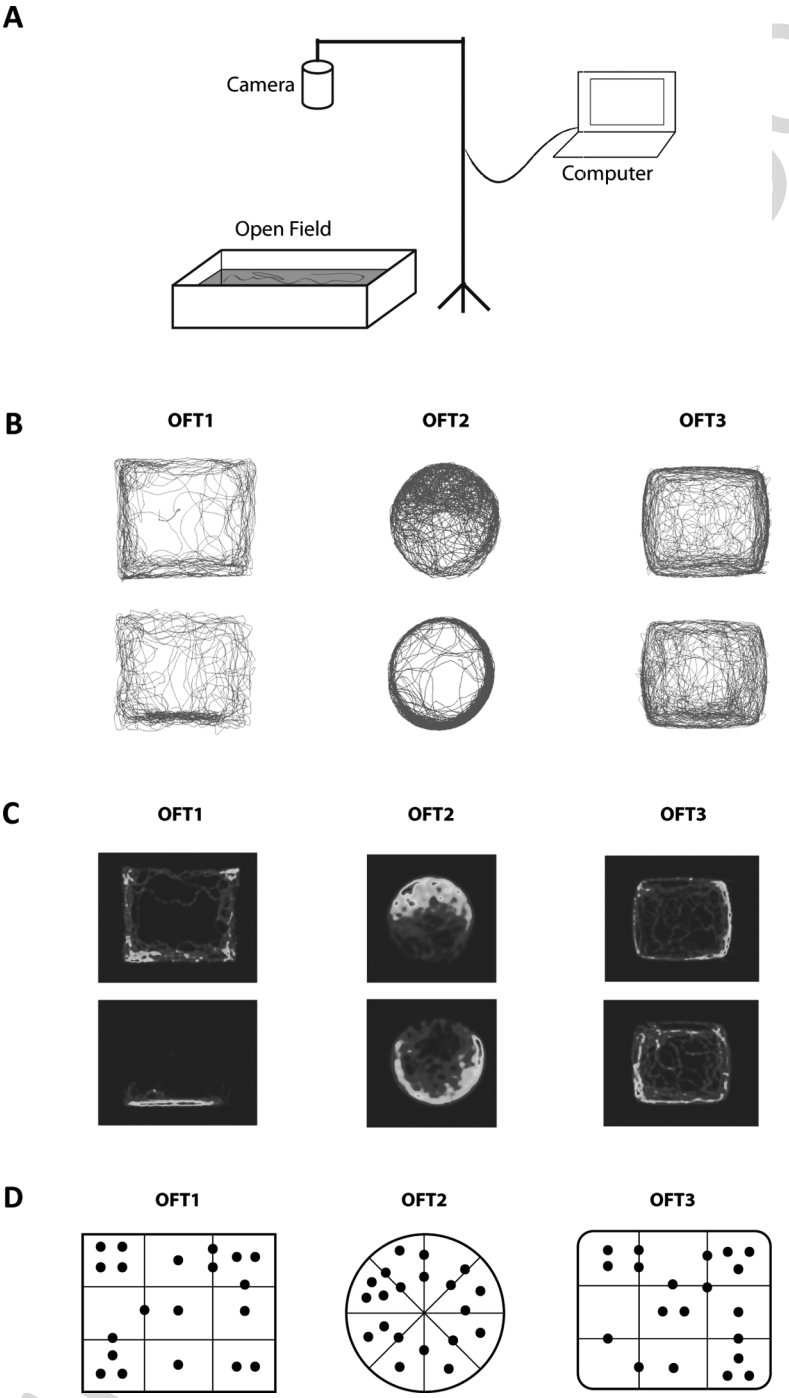

Fig. 12.1. Experimental setup and representative homebase behavior demonstrated in three different 30-min open field tests (OFT1-3). OFT1 was the large rectangular arena, OFT2 was the circular arena, and OFT3 was the small *square* arena. **a**. A typical experimental setup to record zebrafish homebase behavior in the open field test. **b**. Traces generated by Noldus Ethovision XT7 software for three different zebrafish. Note clear spatial preferences of zebrafish OFT behaviors.**c**. Density maps generated for the same fish by Noldus Ethovision XT7 (*see* Section 3.3d for details). D. Summary of homebase topography for all fish ($n = 20$ per OFT) tested here. Each homebase is shown as a *black dot*. Note that each fish was able to establish a clear homebase, typically encompassing one or, less frequently, two sectors (two-sector homebases are shown as *dots* on the border between the two respective sectors).

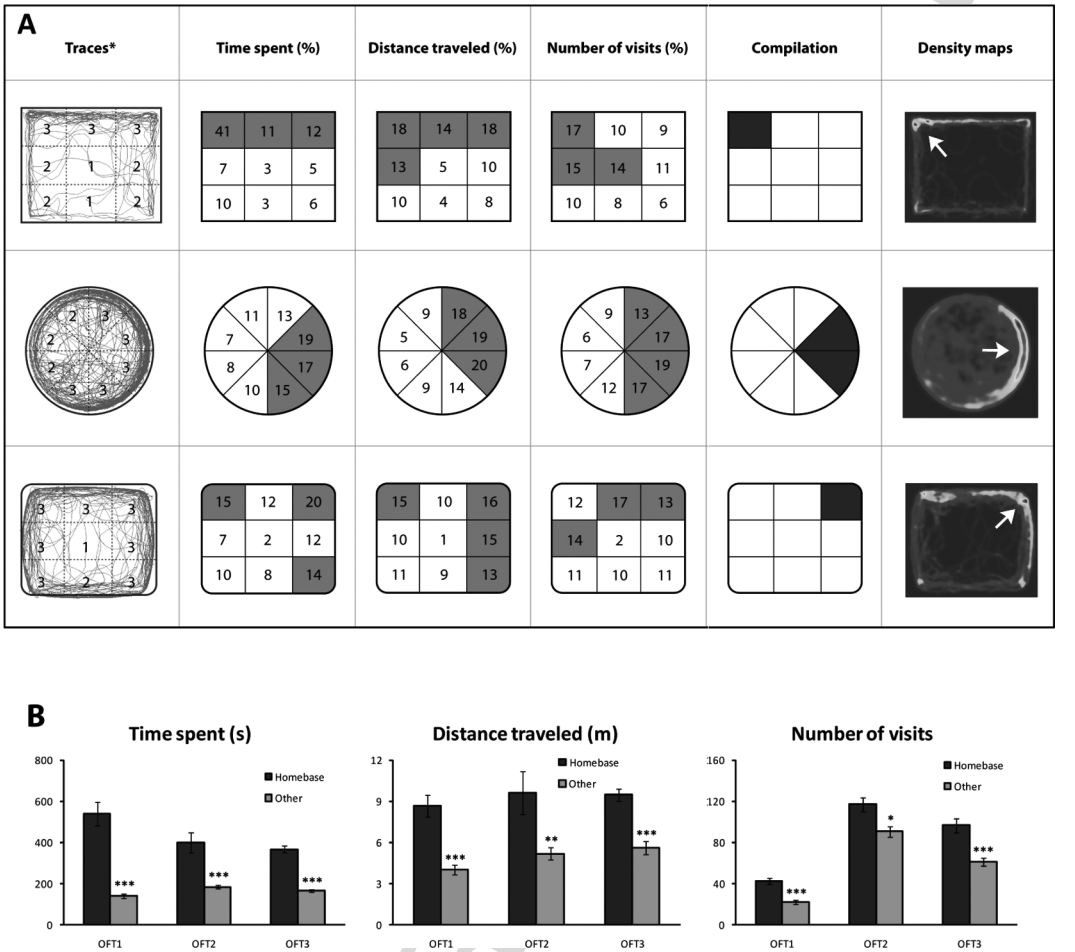

Fig. 12.2. Methodology of homebase identification in three representative zebrafish observed in three different open field test (OFT) tanks for 30 min (see details of the OFT tanks in legend to **Fig. 12.1)**. **a** – Summary of the algorithm used in this study to identify zebrafish homebases. Briefly, the traces were generated by Noldus Ethovision XT7 and scored manually by two experienced observers, using a 0–3 scoring system. Time spent, distance travelled, and number of visits (frequency in zone) were calculated using the video-tracking software for each zone of OFT arenas, and expressed as percent of total. Potential homebases were identified and mapped based on top three percentages. These maps were then compiled to establish the overlap of all four levels of analysis. Density maps were generated by video-tracking software (for time spent data) and used as an additional tool to visualize and reconfirm zebrafish homebase behaviors (indicated by *white arrows*). Note a good correlation between different homebase-related behaviors and their spatial patterning (that enables a precise identification of zebrafish homebases). **b** – Confirmation of zebrafish homebases (identified using the method described above) based on calculation of average time spent, distance traveled, and the number of visits per a homebase sector vs. a nonhomebase sector of the OFT arena. Note striking and highly significant differences in zebrafish behavioral activity between homebase and nonhomebase OFT zones.

differed in size, color, shape, and texture and were selected to reveal differences in zebrafish homebase activity that may be potentially associated with distinct OFT environments. Note, however, that these OFT designs may vary according to the experimenter's preference.

2.3. Experimental Setup

The OFT should be filled with aquarium water to the level of about 12 cm. Apparatuses should rest on level ground with the same distance (e.g., 114 cm, as in our experiments) from the camera (**Fig. 12.1a**). Based on our experience, the standardized 12-cm water level allows enough room for the fish to move freely in the OFT apparatus, yet shallow enough to minimize extensive vertical movements (which may be misdetected by video tracking systems). OFTs should be positioned for optimal lighting while avoiding all glare from the room's light source. Use a light meter (e.g., 840006 by Sper Scientific, AZ) to ensure that all areas of the OFT apparatus are illuminated with the same intensity. Optimal and homogeneous lighting conditions are important for this protocol as shadows could influence zebrafish locomotion. In our experiments, the OFT lighting level was 500–700 lux, as detected by lightmeter applied to 8–9 zones of the novel arena.

2.4. Computer-Aided Analysis

Analysis of recorded trials can be done on- or off-line using commercially available video-tracking software – for example, Ethovision XT7 (Noldus Information Technology, Netherlands); refer to **Chapter 1** by Cachat et al. for more details.

3. Procedure

3.1. Acclimation and Pre-treatment

Transport animals from their holding room to the experimental room for acclimation 1 h prior to testing. The water used in the OFT must be the same temperature as the holding room. If using filtered deionized water drawn from a tap, note that temperature differences can evoke unwanted stress in animals. Therefore, filtered tap water may be drawn the night before, to acclimate to room temperature prior to testing. Alternatively, adjust temperature using hot water.

3.2. OFT Testing

Fill the tank with 12 cm of room-temperature filtered water. Begin video recording and promptly place the fish in the center of the OFT to begin the trial. Video-record for 30 min after placement of the fish. The trial duration may be modified (e.g., increased to several hours) according to researchers' needs and experimental goals. The experimenters should not be present in the room during the time of recording, to prevent disturbances to the fish. After recording, return fish to holding room. When changing water in between trials, make sure to place OFT back in the same place to avoid distorting its position relative to the camera (movement could interfere with proper zone alignment when computer-aided analysis is applied). Also ensure that the

OFT and its environment are as homogeneous as possible. For example, remove any furniture items from the vicinity of the tank (that zebrafish can perceive as additional visual cues). For details on troubleshooting, refer to Notes 1–6.

3.3. Homebase Analysis

1. Transfer the videos to a computer for subsequent analysis using video-tracking software. Divide the OFT arenas into desired zones (**Figs. 12.1d**, **12.2a**) and set event rules to precisely and consistently register behavioral endpoints including time spent (s), distance traveled (m), and the number of visits to pre-defined zones. Fish tracks and density maps can also be generated to visualize zebrafish homebase behaviors based on swimming activity, location, and time spent (**Figs. 12.1b–c**, **12.2a**). For details on troubleshooting, refer to Note 7.
2. Identify zebrafish homebases using the following protocol (also *see* **Fig. 12.2** for details):
   a. Examine traces assigning a score of 0–3 for each zone. A score of 0 denotes no traceable activity within that zone, and 3 corresponding to very high activity. With scoring relative to each individual fish (*see* **Figs. 12.2a** and **12.3** for an example), consider each zone as a potential homebase based on tracing scores of 2 or higher. Note, however, that the score used here can be modified by the investigators. For example, more (or less) elaborate scoring system can be used, if necessary.
   b. Calculate the endpoints of distance traveled, number of visits, and time spent for each individual fish for each zone/sector of the OFT arenas. Express the total 30-min

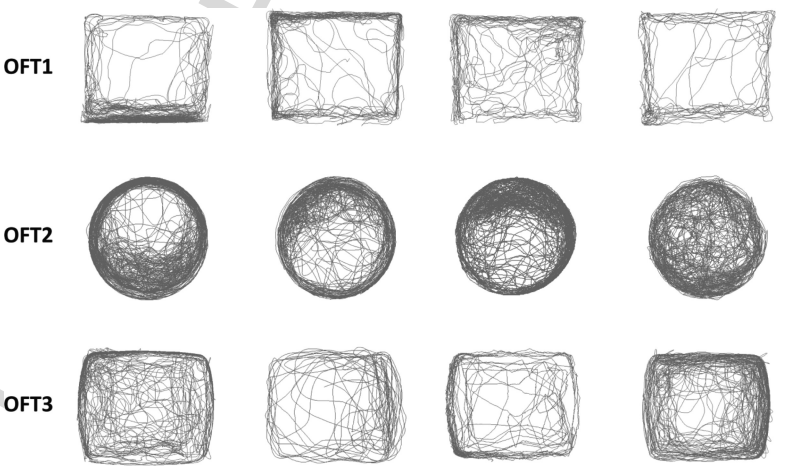

Fig. 12.3. Examples of traces recorded in three different open field tests. Note the individual differences in homebase formation.

activity score for each individual fish for the entire OFT arena as 100%. From this, calculate the percent of activity (of total) for each zone of the OFT. Consider a zone a potential homebase based on three maximal percentages of the total distance traveled, time spent, and number of visits within that zone, as shown in **Fig. 12.2a**.

c. Superimpose these four criteria for each tank in order to identify overlapping zones. In turn, overlap of all homebase-specific loci defines that area as the final homebase for the particular trial.

d. For additional confirmation, generate density maps using Noldus Ethovision XT7, using the *EthoVision Heatmap Generator*, and add-on downloadable through the company's website (http://www.noldus.com/restricted/ethovision-heatmap-generator). Set the time interval equal to that of the recording, to generate a color gradient ranging from yellow to red based on the time spent in location (**Figs. 12.1c** and **12.2a**). This option will usually provide a good method to visualize zebrafish homebase behavior and will strongly correlate with homebase areas detected using either criteria (**Fig. 12.2a**).

For details on troubleshooting, refer to Notes 8–9.

3.4. Statistical Analyses

1. Homebase data can be analyzed using the chi-square (χ^2) or Wilcoxon-Mann-Whitney U-test. The *t*-test can also be used for normally distributed data. The U-test is useful when comparing the behavior exhibited in the homebase vs. the nonhomebase area. The χ^2 test can be performed to analyze the spatial distribution of homebase-related behaviors, comparing *actual* percentages of time spent, number of visits and distance traveled in each zone (of total 30-min scores) with *theoretical* random (by-chance) distribution of these. First, calculate χ^2 data for each endpoint, each OFT tank, and each individual fish. Once all homebases are identified (as described above), generate three combined homebase topographic maps for all three OFT tanks, with dots representing each individual homebase (**Fig. 12.1d**).

   Additionally, the χ^2 test can be applied to compare actual spatial distribution of all homebases (established in the respective OFT) with random by-chance distribution. In all our experiments, significance was set at $p<0.05$. Furthermore, *n*-way Analysis of Variance (ANOVA) can also be utilized. For example, one-way ANOVA is appropriate for comparing homebase behaviors in more than two different OFT types or more than two experimental groups, while one-way ANOVA with repeated measures would be suit-

able for comparing OFT types across test minutes. *N*-way ANOVA can be applied, for example, for the comparison of OFT type, time, drug, dose, sex, etc. Additionally, these analyses must be followed by a post-hoc test (e.g., Tukey or Dunnett tests).

2. To further reconfirm the homebase behavior, assess the average *per zone* activity for homebase-specific (vs. non-homebase) areas, based on percentages of time spent, distance traveled and number of visits, calculated as described above. Use U-test or ANOVA to analyze this data. For details on troubleshooting, refer to Note 10.

4. Notes

1. *Zebrafish homebase formation and/or exploration centered in middle of OFT, or focused nonrandomly on one area of OFT.* Verify that lighting conditions are optimal. For example, use a light meter (e.g., 840006 by Sper Scientific, AZ) to ensure the standard lighting conditions. Record 6–8 points (corner, near walls, center) ten times. If necessary, relocate the OFT to obtain homogeneous lighting data. Glare from ceiling lights can cause a glare that may be aversive, forcing the fish to one particular area. Shadows cast by the positioning in the room or overhanging camera can also attract the fish, and affect their homebase responses. When using opaque arenas, uneven or additional objects near or under the tank can provide cues for the fish. Therefore, visual cues must be kept to a minimum, ensuring homogenous conditions of testing environments.
2. *Zebrafish display aberrant behavioral phenotypes*
   Several factors due to strain variation may nonspecifically affect animal behavior. For example, low- and high-anxiety zebrafish strains may display higher or lower baseline anxiety levels. Some of these phenotypes could therefore result in a modulation or ablation of homebase behavior. To rule out such nonspecific factors, a careful examination of zebrafish neurological and sensory phenotypes is recommended.
3. *Fish display excessive freezing or little locomotion*
   The presence of the experimenter in the room during testing may startle the fish, causing a heightened anxiety-like behavior. Also, differences in water temperature or excessive net stress prior to testing can also induce a state of decreased locomotion. Higher anxiety strains, such as the leopard strain (12), may also demonstrate decreased exploratory behavior.

4. *High variability of observed responses*
   Despite animals' inherent tendency to form homebases, high variability in observed responses is common in behavioral research. This may be explained by genetic influences or animal stress in the animal facility (improved husbandry could normalize zebrafish behavior). It is also important that the testing room conditions (temperature, soundproofing, lighting, etc.) be carefully controlled in the experiments. Additionally, an increase the sample size could normalize aberrant results (based on our experience, significant zebrafish data can be obtained for $n = 20$ per group). Since many studies currently involve a battery of tests, this could also influence OFT performance. Use less stressful challenges before subjecting the fish to the OFT. Acclimate fish for at least 7 days before the tests as well. Excessive stress may create potential confounds. For instance, increased freezing may increase the duration of time spent in a particular area, but will not be indicative of a homebase.
5. *Role of memory and conditioned responses*
   Zebrafish show good learning and memory capacities, can recall training for up to 10 days (30), and display robust intra- and inter-session habituation (11). Because of this, re-testing zebrafish in a novelty-based paradigm such as the OFT should be avoided (refer to **Chapter 1** by Cachat et al. for more details). However, since the OFT invokes a robust behavioral phenotype in zebrafish, this test may be utilized to further dissect the effects of various experimental manipulations on anxiety and spatial memory.
6. *Fish leap out of OFT during trial*
   Some fish have the tendency to slowly meander up to the edge of the tank and subsequently "catapult" themselves out of the OFT. Thus some experiments may necessitate that the water level be filled to the top of the OFT, in which case the loss of fish is unavoidable. Precautions to deter the fish (e.g., mesh wire over the OFT) may provide confounding cues. However, keeping a water level several centimeters below the OFT edge will generally prevent this problem.
7. *Software not detecting fish*
   This lack of object detection can be resolved by altering one or several setting as well as ensuring adequate lighting (*see* **Chapter 1** by Cachat et al. in this book for details).
8. *The endpoint of duration in zone does not correspond to the traces*
   Traces are representative of the path taken by the zebrafish. Therefore, a significant duration within a zone may not

necessarily correspond to movement, but rather a prolonged bout of immobility (freezing), which would appear as a single, unnoticeable point on the trace map.

9. *Zebrafish appear to be forming homebases (through track analysis), but the behavior is not significant when endpoints are evaluated on a per zone basis*
The zone sizes may be too large. For example, the OFT may be better divided into nine smaller zones instead of four large quadrants. Even more zones may be needed as the size of the OFT increases (due to the fact that zebrafish homebase size may remain the same despite an enlargement of the arena).

5. Anticipated Results

Using this protocol, the fish are expected to establish distinct homebases – particular areas where they spent most of the time, traveled more, and visited most frequently (**Figs. 12.1b–c**, **12.2** and **12.3**). These homebases will most likely be located near the walls of the tanks, and usually consist of one (less frequently – several) homebase zones (**Fig. 12.1d**). Spatial distribution of the

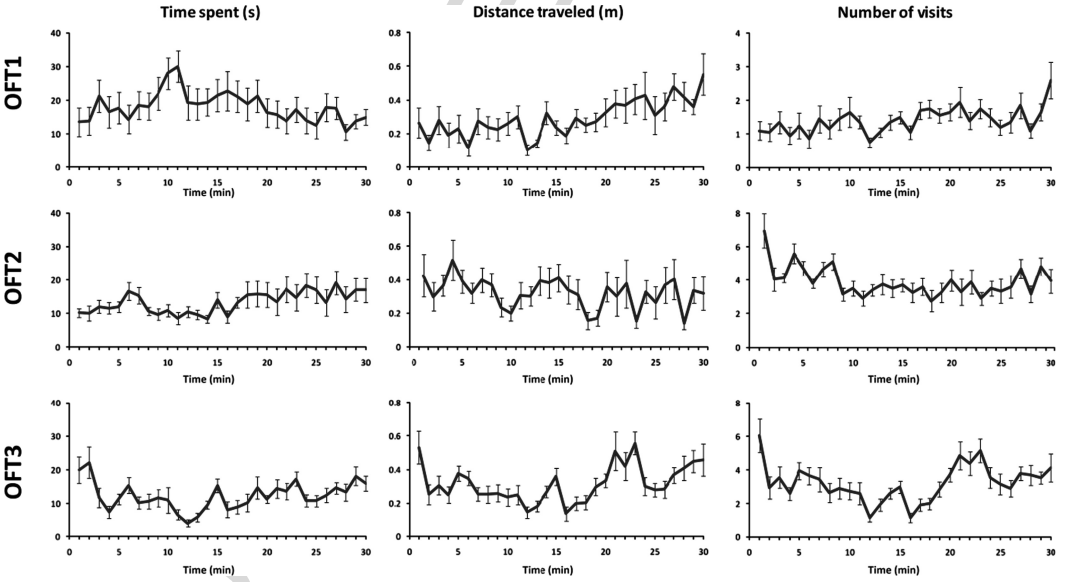

Fig. 12.4. Temporal dynamics of zebrafish homebase behaviors in three different open field tests for 30 min (distance traveled, time spent, and frequency of visits). Homebases were identified using our protocol (**Fig. 12.2a**) and reconfirmed, as shown in **Fig. 12.2b**. Note that zebrafish maintain active presence in their homebases throughout the test.

time spent, distance traveled, and number of visits are expected to show significant differences in the homebase relative to the area outside the homebase. The "combined" analyses of topographical maps of zebrafish homebases in each of the three OFT tanks (**Fig. 12.1d**) will show that the different OFT zones are chosen *at random* by different zebrafish for their homebases, without spatial preference of homebase location in relation to a particular OFT (**Fig. 12.3**). Furthermore, comparison of the distance traveled, frequency of visits, and time spent within the homebase zones would reveal similar temporal dynamics of homebase behavior across different OFT arenas. Essentially, zebrafish will generally maintain constant levels of activity in their homebases (**Fig. 12.4**), frequently visiting these strategic loci.

6. Summary

Here we described a simple method to identify and phenotype homebase behavior in zebrafish. Zebrafish homebase behavior (**Fig. 12.1–12.4**) is not determined by innate features of the OFT novelty, but rather actively established by animals exploring their environment, strikingly resembling homebase behavior in rodents (24, 31, 32). This new paradigm may also have a variety of important potential applications in biomedical research. For example, homebase analyses may be useful for screening pharmacological agents in zebrafish, since this behavior has already been demonstrated to be affected by different drugs in rodents (e.g., (28)). Furthermore, such analyses can be suitable for testing various inbred and mutant zebrafish strains, which may display aberrant behaviors including altered homebase phenotypes. Homebase behaviors are also highly relevant to exploration and cognition, and zebrafish models with abnormalities in either domain are likely to have impaired homebase behavior.

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Chapter 13

Neurophenotyping of Adult Zebrafish Using the Light/Dark Box Paradigm

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Abstract

The light/dark box test, traditionally used to quantify rodent anxiety-like behavior, has recently been applied to the adult zebrafish (*Danio rerio*). Utilizing the fish's scototaxis (aversion to bright areas and natural preference for the dark), this paradigm can be used to assess levels of anxiety in adult zebrafish. The light/dark box is a simple and time-efficient one-trial test that does not require pre-training the animals. Importantly, this novelty-based paradigm may also represent a useful tool for studying the pharmacological modulation of zebrafish behavior. Summarizing the experience with this model in several laboratories, here we outline a protocol for the neurophenoptyping of zebrafish anxiety-like behavior using the light/dark paradigm.

Key words: Zebrafish, Light/Dark box, Scototaxis, Anxiety, Novelty-based paradigm.

1. Introduction

Various novelty-based paradigms, some of which are comprehensively covered in this book, have been developed to quantify zebrafish behavior (1–7). The light/dark paradigm, traditionally used in animal (rodent) behavioral research (8–11), has only recently been applied to zebrafish (12–15). Nevertheless, this test, based on the innate fish preference for the dark (scotophilia or scototaxis), is receiving a growing popularity in neurobehavioral laboratories (2, 14, 16, 17).

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Previous research in rodents has shown that while anxiolytic manipulations can facilitate exploratory activity (i.e., increased entries and duration in the light part), anxiogenic drugs cause the opposite effect (8, 9, 11, 18). Given the amazing translatability of zebrafish models into rodent and human neurophenotypes (1, 2, 19), the possibility to adapt a scototaxic paradigm to zebrafish was logical (*see* (15) for details). Prior evidence has shown that scototaxis may contribute to predator avoidance in nature, as adult zebrafish stand out clearly when swimming amidst a light background. This further underscores their inherent anxiogenic response evoked when confined to a white background (12).

Several modifications have been made to produce a zebrafish paradigm that parallels the rodent light/dark assays (2, 12, 17). The utility of the zebrafish light/dark box is further strengthened when used in conjunction with video-aided analysis, which can assist in tracking and quantifying animal behavior. Here we describe a simple protocol for using the light/dark model to assess stress- or drug-evoked alterations in adult zebrafish anxiety.

2. Methods and Materials

2.1. Animals and Housing

Adult zebrafish (e.g., wild-type short-fin, 6–8 month-old; ≈50:50 male:female ratio) can be obtained from a local commercial distributor, and should be given at least 20 days to acclimate to the animal facility. Animals can be housed in groups of approximately 20–30 fish per 40-l tank. Tanks should be filled with deionized water, with both room and water temperatures maintained at ≈25°C and water pH at 7.0–8.0. Illumination can be provided by ceiling-mounted fluorescent light tubes (e.g., 1000 lux) on a 12–12 or 10–14 h cycle, consistent with the zebrafish standard of care (20).

2.2. Apparatus

Several modifications of the light/dark paradigm, used by our laboratories, will be discussed here. One modification, used at Tulane University, USA (Modification I), represents a rectangular Plexiglas tank (15 height × 30 length × 16 cm width) that rests on a level surface, and divided into two equal vertical portions (**Fig. 13.1a**), demarcated by black and white coloration (2). It differs from the rodent apparatus in that it is sealed to prevent leakage, filled with water to a height of 12 cm, and does not have a wall (with a sliding door) between the compartments. In this modification, fish can freely swim between the light and dark compartments of the apparatus.

Another, more sophisticated, modification of this test was successfully used by Brazilian laboratories (Modification II,

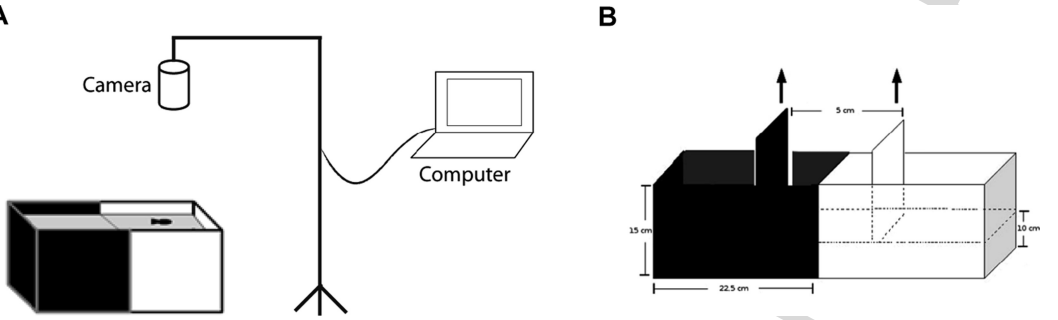

Fig. 13.1. The light/dark paradigm for characterization of adult zebrafish behavior. **a** – Typical experimental set-up used in Tulane University, USA (Modification I), allowing for video-recording for subsequent analysis using video-tracking software. Note the camera need only be centered above the *white* half, as the *black* half will not be analyzed. **b** – Typical light/dark box test used in Brazilian laboratories (Modification II, as described in (13)).

Fig. 13.1b). This modification, applied to zebrafish and some other fish species (13, 15), represents an acrylic tank of equal measures (15 height × 45 length × 10 cm width) with half black/half white walls and bottom colored, and filled with water to a height of 10 cm. The colored material chosen should be non-reflective, in order to avoid the tendency of animals to behave in relation to their own reflection. Unlike Modification I, this apparatus contains sliding central doors, colored with the same color of the aquarium side, thereby defining a central compartment with 15 height × 10 length × 10 cm width (**Fig. 13.1**).

During experiments, the tank must be rotated after each trial, so as to eliminate orientation effects. The tanks are illuminated by environmental light (e.g., by a 60-W light bulb, located at 1.80 m above the tank top), which kept illumination uniform and constant between trials (**Fig. 13.1**).

2.3. Experimental Setup

The light/dark box should be positioned for optimal lighting while avoiding all glare from the room's light source. Since the brightness of the apparatus is a fundamental feature of this paradigm, use a light meter (e.g., 840006 by Sper Scientific, AZ) to ensure that all areas of the apparatus are illuminated with the same intensity. For a light-sensitive assay such as this, optimal and homogeneous lighting conditions are important for this protocol. The results are also sensitive to the light amounts; animals tested under low-light levels (250 lux) spend more time in the white compartment than animals tested under high-light levels (500 lux) (**Fig. 13.2**). Additionally, unlike other behavioral tests, manual scoring is complicated by the nature of this apparatus, as the experimenter would have to lean over the apparatus to gather the data. This can be problematic for the testing, since the experimenter could cast a shadow or startle the fish. However, the use of a webcam and computer can alleviate this problem, as it allows

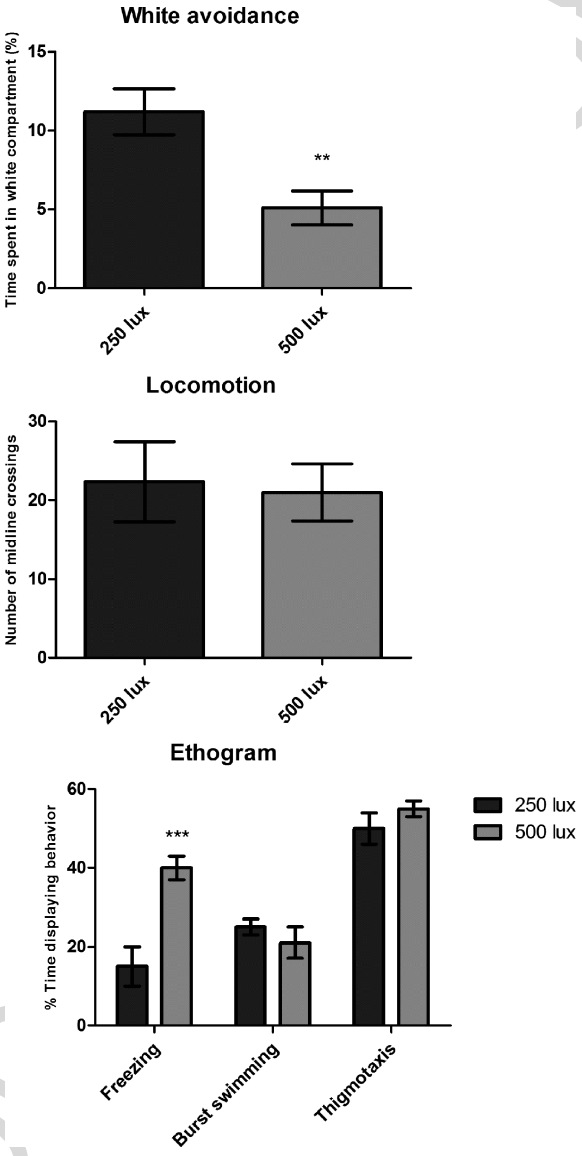

Fig. 13.2. Lighting levels alter the results of the zebrafish light/dark box test. Animals tested in Modification II of this test for 15 min under high illumination levels (500 lux) spend less time in the white compartment and freeze more while there ($n = 10$), Maximino et al., unpublished data; ***$p<0.001$, **$p<0.01$.

for remote observation via the computer screen, as well as allows option for later video-aided analysis (**Fig. 13.1a**).

2.4. Behavioral Endpoints

Behavioral scoring can be performed manually to quantify the latency to enter (s), time spent (s), average entry duration (s), and the number of entries to the white half of the apparatus (due to the dark background, zebrafish behavior in the black compartment cannot be detected, and, therefore, is not assessed in this paradigm). To further characterize zebrafish light–dark

preference, the white:total time spent ratios can be calculated for both cohorts. Video-tracking programs, such as Ethovision XT 7 (Noldus Information Technology, Netherlands; *see* Chapter 1 in this book), can also be used to analyze variety of additional endpoints, such as distance traveled, velocity, meandering, turning angle, angular velocity, or time spent moving.

3. Procedure

3.1. Acclimation and Pre-treatment

Transport the animals from their holding room to the experimental room for acclimation 1 h prior to testing. During this time, if the study involved pharmacological manipulations, prepare 3–4 L beaker(s) in order to administer the drug via immersion. Fill each beaker with ~3 L of exposure solution, maintained at the same temperature as the holding room (drug concentration is determined by referring to prior literature and/or pilot study). After the acclimation period (and when the drug is fully dissolved), the fish are individually transferred to the exposure beaker filled and treated for the optimal exposure time (lengths of treatment will vary with the drug, but is generally in intervals of 10, 20, or 30 min).

3.2. Light/Dark Box Testing

Fill the light/dark apparatus with 10–12 cm of room-temperature filtered water. After the necessary pre-treatment time has elapsed, begin video-recording and carefully move the fish to the light/dark box. If using Modification I, introduce the fish into the black half (facing the wall), and video-record for 6 or 10 min, while manually scoring the behaviors. Recording times may be extended, however, 6- or 10-min trials appear to be optimal for most experiments. If using Modification II, introduce the fish by netting it from the maintenance tank and transferring it, as quickly as possible, to the central compartment; in this case, keep the sliding doors on for 3–5 min, for acclimation, then remove them to allow the animal to explore the apparatus. Standard 15 min testing sessions have been used for this modification. If endocrine data are collected, euthanize the fish by immersion in 500 mg/L Tricaine (*see* Chapter 11, this book). Store each fish individually in Eppendorf tubes, denoting its treatment group and store at –80°C for later cortisol extraction. For details on troubleshooting, refer to Notes 1–3.

3.3. Video-Aided Analysis

As already mentioned, zebrafish behavioral endpoints may be evaluated using video-aided analysis. Transfer videos to computer for subsequent analysis using video-tracking software. Define the arena to overlap with the outline of the apparatus, and define the zone to encompass only the white portion of the test. Accord-

ingly, set the program to track objects that are darker than the background. In addition to evaluating the endpoints recorded manually, other indices can be specified to include the time spent (s) in the white zone, distance (m) traveled, velocity (m/s), and immobility (freezing) frequency and duration. Traces of the path taken by the animal can also be generated (**Fig. 13.3a**; refer to chapters on visualizing and video-tracking zebrafish behavior in this book). For details on troubleshooting, refer to Notes 4–7.

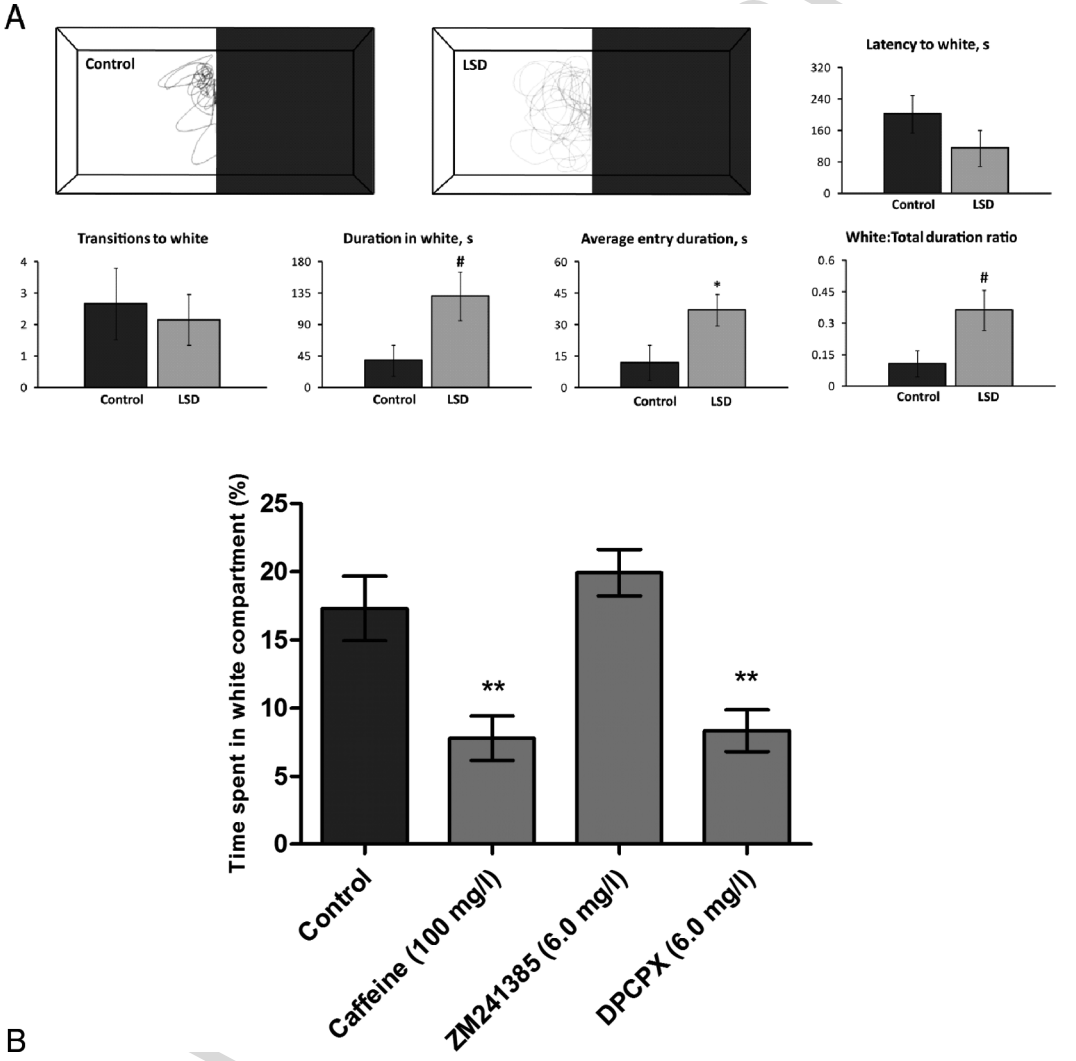

Fig. 13.3. Behavioral effects of selected pharmacological agents in the light/dark box test. **a** – effects of Lysergic acid diethylamide (LSD) (250 μg/L) on zebrafish tested in the 6-min light-dark box test, Modification I ($n = 12$) (data are based on (14)). Representative traces were generated by Ethovision XT7 software using the top view video-recording; only light part of the box and a small part of the dark part are shown in this panel. **b** – effects of several adenosine receptor antagonists (caffeine, nonselective antagonist, 100 mg/L; ZM241385, A_{2A} receptor antagonist, 6 mg/L; DPCPX, A_1 receptor antagonist, 6 mg/L) on zebrafish tested in a 15-min test (Modification II); $n = 12–14$; Maximino and Herculano, unpublished data; $^{**}p<0.01$, $^{*}p<0.05$, #p=0.05–0.1 (trend) vs. control.

3.4. Statistical Analysis

Use the Mann-Whitney *U*-test for comparing two groups. Student's *t*-test may be used for normally distributed data. Our group has devised a useful template to calculate statistics and generate graphs for zebrafish manual or video-tracking data, which can be downloaded from our laboratory's website at: www.kaluefflab.com/science.html. For more than two groups, use an Analysis of Variance (ANOVA), followed by an appropriate post-hoc test (e.g., Tukey, Dunn, Newman-Keuls, or Dunnet tests). In general, *n*-way ANOVA may be applied, with commonly used factors being: treatment, dose, sex, strain, time, trial, or age.

4. Notes

1. *Zebrafish display atypical and/or varied behavioral phenotypes* Different zebrafish strains can have varying baseline levels of anxiety (1), which could result in the failure to cross into the white half of the apparatus. Alternatively, it may represent a behavioral hyperactivity, or disinhibition to regard the white half as aversive. Sensory deficits, such as impaired vision, will also produce atypical data in this test. Likewise, altered cognitive functions will produce abnormally low (good memory) or high (poor habituation) exploration of the white area. Finally, variations in responses can also be seen among the standard wild-type strain, with both low- and high-avoidant fish often present in the same cohort (21). Low-avoiding fish can be particularly problematic because of their heightened tendency to quickly habituate to the white half of the tank. In general, to rule out nonspecific factors, a careful examination of zebrafish cognitive, neurological, and sensory phenotypes is recommended in case if atypical behavioral responses are observed in the light/dark paradigm. Additionally, the time of the trial may have to be adjusted to obtain more reliable data (*see* above).

2. *Fish consistently fail to cross into the white half during the trial* Generally, the presence of the experimenter in the room during testing may startle the fish, causing a heightened anxiety-like behavior, especially if a webcam and computer setup are not employed. Also, differences in water temperature or excessive net stress prior to testing can also induce a state of decreased locomotion. Furthermore, after ruling out strain variation (*see* above), the pharmacological agent itself may need to be considered. For instance, some fish may often remain in the dark half for an entire 6-min trial. If they are treated with an anxiogenic drug, which leads to an even greater aversion to cross into the white, the drug-

evoked effects will be masked by high background anxiety (floor/ceiling effect). To compensate, consider extending the trial duration (e.g., to 30 min), which will encourage more active animal exploration.

3. *Fish displaying abnormally high thigmotaxis*
   Fish spending too much time (~30% of the total test time) clinging to a particular wall (or to walls in general) of the apparatus (maximum distance of ~2 cm from the wall) may be responding to its own reflection (15). Consider changing the material of which the apparatus is made of to avoid confounding variables. The experimenter should keep track of the thigmotactic fish, and thigmotaxis itself should be analyzed (either by recording its frequency and duration in individual fish, or by recording the number of fish that displayed it) (15).

4. *Software not detecting fish*
   This lack of object detection can be resolved by altering one or several setting as well as ensuring adequate lighting. Notably, it is essential that the subject be defined as darker than the background (*see* Chapter 12 for details).

5. *Fish freezes in white compartment, after first choice*
   Occasionally, fish can freeze after they choose the white compartment, no longer exploring the apparatus for the whole trial duration. This is especially common for stressful manipulations; or if there is noise, vibration or movements in the experimentation room. Data from this animal should be discarded from analysis. The experimenter may keep track of the freezing fish, and freezing behavior itself should be analyzed (either by recording its frequency in individual fish or recording the number of fish which froze) (15). However, careful attention should be paid to the frequency/patterning of this behavior to ensure external factors (discussed above) are not inducing excess freezing.

6. *Software not producing data on fish*
   Verify the detection settings and ensure that the software is able to track the fish in the white half of the tank. However, it is most likely that the fish for these particular trials did not cross into the white half during the trial (this is especially common among control cohorts).

7. *Fish jumping out of the tank*
   Infrequently, the animal "jumps" out of the test tank. When this occurs, the experimenter must rapidly pick up the animal and discard it. Behavioral data from such fish should be excluded from the analyses (15).

5. Anticipated Results

The observed behavioral responses and indices of zebrafish anxiety assessed in the light/dark box test should generally parallel those observed in the novel tank and open-field models. However, some differences in pharmacological results with variations of these tests have been observed, suggesting that these models may target different aspects or subtypes of anxiety (6). In line with this, anxiety levels can be attenuated or exaggerated depending on drug exposure. For example, exposure to anxiolytic agents will cause an increase in transitions to and time spent in the white half of the tank. A decreased latency to cross into the white half should also be expected. Although not specific to this apparatus, the bouts and duration of freezing, as well as erratic movements, should also be decreased by a reduction in anxiety (**Fig. 13.3**). Conversely, the opposite is expected with the administration of anxiogenic compounds. For example, acute treatment with methylmercury is anxiogenic in zebrafish, as assessed by light/dark preference (**Fig. 13.4**).

In addition to pharmacological modulation, other manipulations can be used in this model. For example, rearing in enriched environment for 2 months increases the time spent in the white compartment of the test tank, compared with those reared in an impoverished environment (15). Thus, positive stress-reducing factors such as environmental enrichment can decrease zebrafish anxiety-like behavior in this test, strikingly paralleling similar findings in rodents (15, 22–24).

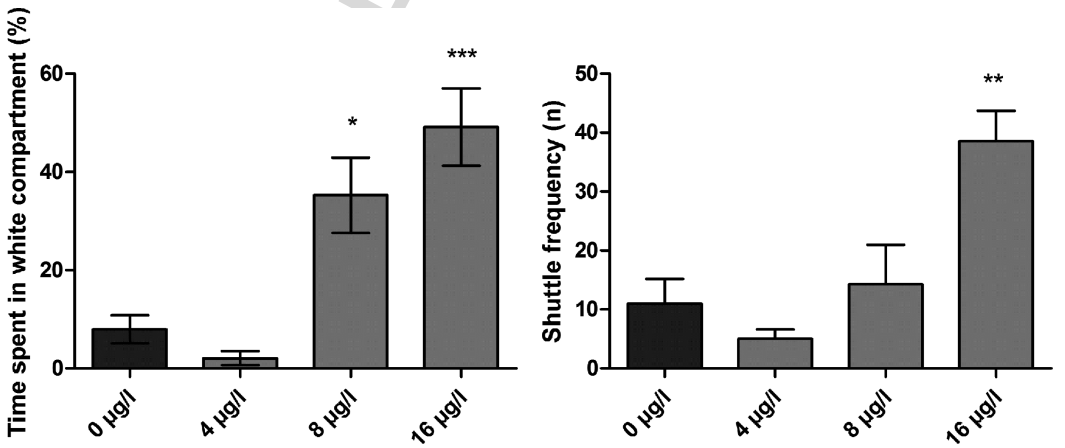

Fig. 13.4. Effects of methylmercury chloride exposure (4, 8, and 16 μg/L for 24 h) on the time spent in the white compartment and total locomotion in the 15 min light/dark box (Modification II) in adult zebrafish ($n = 10–14$). Maximino et al., unpublished data; ***$p<0.001$, **$p<0.01$, *$p<0.05$ vs. control.

6. Summary

The light/dark box test is emerging as a promising behavioral assay to quantify anxiety-like behavior in adult zebrafish. Overall, this test serves as a useful addition to the array of novelty-based paradigms, being unique in its ability to assess light/dark aversion. Importantly, the quantification of scototaxis may serve as a reliable tool in neurophenotyping research and high-throughput drug screens. Rodent literature has demonstrated that the light/dark test is especially useful for phenotyping mutant strains, a utility that has recently been confirmed in zebrafish (21). However, the evaluation of different strains using this paradigm has yet to be undertaken.

In addition to its use in adult zebrafish, the light/dark paradigm has recently been applied to larvae, although in a different model. Notably, unlike adults, larval zebrafish are *phototactic*, as they prefer lighter areas (25) and move toward well-lit areas when presented with a choice (26). As such, larvae locomotion patterns have been studied under a range of lighting conditions with varying durations. For example, when subjected to an extended period of darkness, larvae locomotor activity is high at first and then decreases to a low level. In an extended light duration, their activity gradually increases to a stable level, but can be also be pharmacologically modulated in both light and dark conditions (27). Logically, the "reversed" light/dark box test could be developed for larvae, and further research is needed in this field.

Overall, the light/dark box is a simple and fast one-trial test that does not require pre-training the animals. This paradigm offers a promising and sensitive tool to complement the other tests measuring anxiety-like behavior in zebrafish.

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Chapter 14

Intraperitoneal Injection as a Method of Psychotropic Drug Delivery in Adult Zebrafish

Adam Stewart, Jonathan M. Cachat, Christopher Suciu, Peter C. Hart, Siddharth Gaikwad, Eli Utterback, John DiLeo, and Allan V. Kalueff

Abstract

Zebrafish behavioral phenotypes are often evaluated in response to pharmacological modulation by various psychotropic drugs. An important step in this process is the method of drug administration. While the most popular drug administration technique in zebrafish research is by immersion, systemic intraperitoneal injection is another effective alternative. This method is useful for drugs that are difficult to dissolve in water, or which require a better control over the amount of drug delivered to an individual animal. Here we outline a simple protocol for the intraperitoneal injection of drugs in adult zebrafish.

Key words: Zebrafish, intraperitoneal injection, drug exposure, drug administration method, anxiety.

1. Introduction

Zebrafish exhibit robust behavioral phenotypes, which can be examined in simple and reliable assays for drug screening (1–4). Our group has made extensive use of these paradigms, often in conjunction with video-aided analysis, to correlate the behavioral and endocrine indices of anxiety-like behavior evoked by psychotropic drug exposure (1, 5–7) (**Fig. 14.1**).

One of the most important steps in using pharmacological agents to study animal behavior is the method of drug exposure (8–10). Indeed, a proper uniform administration of the chosen drug is crucial to the outcome of the study. In zebrafish, drug exposure is usually performed via immersion in a drug-containing

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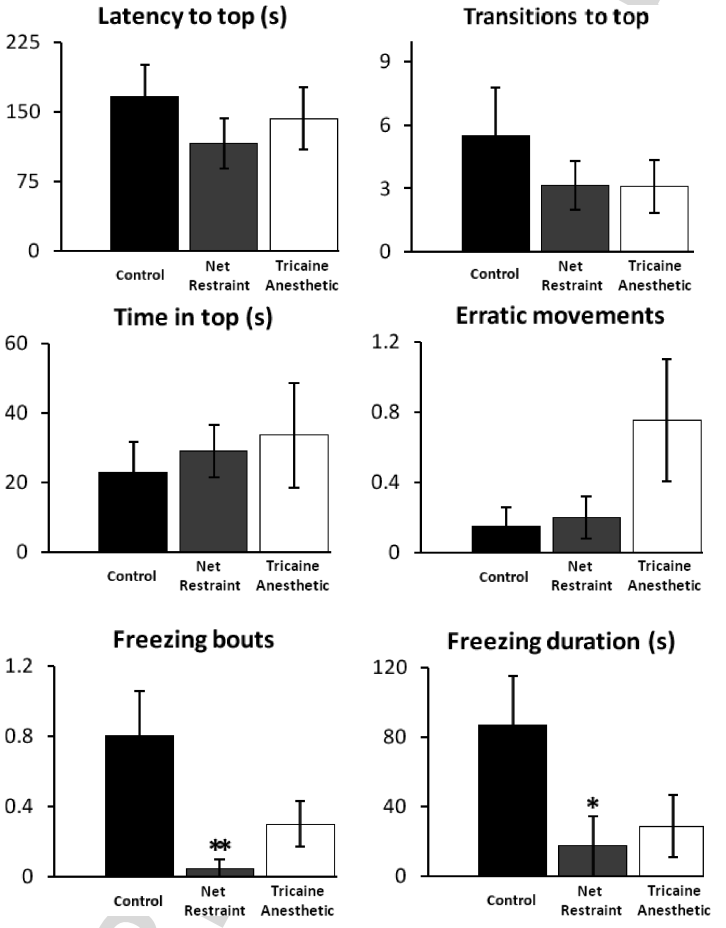

Fig. 14.1. Behavioral data comparing controls (water immersion only) and intraperitoneal (i.p.) injection (via net immobilization) with i.p. injection using Tricaine anesthesia ($n = 13–14$). * $p<0.05$, ** $p<0.01$, vs. control.

solution for a specified duration (6, 7, 11, 12). However, certain drugs do not readily dissolve in water and can therefore be arduous to administer (13).

An alternative approach is to administer the drug via systemic intraperitoneal (i.p.) injection. This is a procedure that, when performed correctly, can be a viable and effective technique for drug delivery. Intraperitoneal administration has been long used for big fish species, such as rainbow trout, Atlantic halibut, tilapia, and crucian carp (14–17), as well as in smaller fish, such as goldfish (18–22), minnows (23–25), and guppies (26–28). Systemic i.p. injections have also been used in several published zebrafish studies (29–33). For example, zebrafish have been used to model the effects of environmental toxins implicated in the pathogenesis of Parkinson's disease, with varying doses of MPTP and MPP+ being administered i.p. (29). Furthermore, i.p. injection has also been

used as an effective administration route to study the reinforcing properties of drugs of abuse in zebrafish (33).

Somewhat more stressful for animals (than immersion), systemic i.p. injections are usually needed when the immersion method of drug delivery is infeasible. While some agents, such as diazepam or 1,3,5-Trinitroperhydro-1,3,5-triazine (RDX), are insoluble in water, but can be dissolved in an acceptable alternative solvent. However, the solvent must be conducive to the health of the fish, as well as have no known reactivity with zebrafish behavior. Again, an additional "solvent" control group must be added to the experimental design. In cases where these two criteria are not met, i.p. injection becomes a viable alternative (34, 35). Whereas i.p. administration is a more precise method than immersion, it is also often the preferred method of administering expensive or rare drugs (36), as well as drugs affecting sideline receptors (*see* (37) for details about the role of administration precision and receptor interaction). Likewise, administration of small volumes of oily substances (e.g., some steroid hormones or similar hydrophobic agents) via i.p. injection may be the preferred method of drug delivery. Other situations where i.p. injection may be preferred involve drugs that can irritate gills (38) or agents that are highly unstable in water (39). Here, we outline a protocol utilizing i.p. injection for drug delivery in *adult* zebrafish for their subsequent testing of a variety of behavioral assays.

2. Potential Limitations

There are also several limitations of using i.p. injection. For instance, it is often necessary to use anesthetics when carrying out the procedure, which may have undesirable effects on the examined behavior and physiology (40). Likewise, age, sex, strain, previous drug exposure, and even time of day of exposure can have important impacts on anesthetic drug responses in various animals, including rodents and fish (41). Another limitation of this method is that it involves considerably more skill (relative to immersion), as care and precision are needed to avoid puncturing the animal's organs, as well as to minimize behavioral anomalies induced by pain (40, 42) (*see* Note 1). Likewise, the procedure requires more time than the immersion method. Furthermore, the i.p. injections can only be performed using small volumes of the drugs (e.g., 5 or, less preferably, 10 μl), and hence, this method may not be appropriate for applying high doses of certain drugs (which would require higher injection volumes). Finally, while the immersion method can be used for chronic drug

administration, *repeated* i.p. injections cannot be performed in small animals such as zebrafish.

3. Methods and Materials

3.1. Animals and Housing

Adult zebrafish (e.g., 6–8 month-old; ≈50:50 male:female ratio) can be obtained from a local commercial distributor, and housed in groups of approximately 20–30 fish per 40-L tank. Tanks should be filled with filtered water, with room and water temperatures maintained at ≈25°C and water pH at 7.0–8.0. Illumination can be provided by ceiling-mounted fluorescent light tubes on a 12–12 or 10–14 h cycle, consistent with the zebrafish standard of care (43).

3.2. Equipment

1. Small 5–10 μL Hamilton syringe (e.g., Hamilton Company, Reno, NV, USA).
2. Net (for immobilizing zebrafish) (e.g., Fisher Scientific, Pittsburgh, PA, USA).
3. Treatment beaker for Tricaine solution.

4. Procedure

4.1. Acclimation and Pre-treatment for Intraperitoneal Injection

1. Transport the animals from their holding room to the experimental room for acclimation 1 h prior to testing. After acclimation, the fish will be individually treated with the chosen drug via i.p. injection. Importantly, this must be organized in intervals of ~10 min to correspond to the time allotted per each 6-min trial, with a ~4 min left-over for preparation for the next one.
2. To administer the drug, anesthetize the fish by immersion in Tricaine (100–120 mg/L; Sigma-Aldrich, St. Louis, MO) for ~30–60 s, until only the gills are moving. Slightly tap on the beaker to see if the fish is still capable of movement to ensure that it is fully anesthetized. Do not leave the fish in the Tricaine longer than necessary, as this is a time-sensitive procedure, and death can result if exposure is prolonged by as little as an additional ~20–30 s.
3. Remove the fish from the Tricaine and lay it down on a sterile surface, turning the animal so its ventral side is facing upwards.

4. Quickly inject 5–10 μL of the drug solution into the peritoneal cavity using a small Hamilton syringe. Note that control fish must be treated by injecting an equal amount of vehicle (e.g., saline or water) solution. The site of injection is in the midline cranial to the base of the pelvic fin. For a general reference, the place of injection should lie about 1 "fin-length" ahead of the pelvic fin base. For details on troubleshooting, refer to Note 1.
5. Move the fish to a 3–4 L holding beaker filled with ~3 L water for the desired pre-treatment time (which, like the dosage, should be determined by a prior literature search or calculation from previous human or rodent studies).

4.2. Behavioral Testing

Fill the apparatus with the specified amount of room-temperature filtered water. After the necessary pre-treatment time has elapsed, begin video recording, and proceed to carefully move the fish to the apparatus. For details on troubleshooting, refer to Notes 2–3.

4.3. Endocrine Analysis

Once all of the behavioral data has been collected and analyzed, a comparison of the cortisol levels between the control and experimental groups can be performed (*see* Chapter 11, this volume). This will allow for the behavioral phenotypes to be paralleled with their respective physiological measurements of anxiety.

4.4. Data Analysis

If a control and single experimental groups were used, utilize the Mann-Whitney *U*-test for comparing these two groups (Student's *t*-test may be used for normally distributed data). If more than one drug dosage was applied, use an Analysis of Variance (ANOVA), followed by an appropriate post-hoc test, such as Tukey, Dunn, Newman-Keuls, or Dunnet tests.

5. Notes

1. *Death results from the procedure.* While general care is needed throughout the procedure, it is most vital to avoid piercing the animal's vital organs during injection. The needles should be only long enough to penetrate the abdominal wall, otherwise one can easily inject into the abdominal organs causing injury. However, an alternate factor to consider is the duration spent in the Tricaine during anaesthetization. Leaving the fish in the solution for too long can be fatal. During the exposure, check for subtle gill movement to rule this out.
2. *Observed anxiety levels are unusually high.* Careful handling of the fish during injection is crucial. If the anesthesia is not

administered properly, or the injection is done roughly, pain and a heightened state of anxiety can result. This may last well into the trial, thereby affecting the observed behavior as well as cortisol levels. Drug and humor leakage in injected fish is not uncommon, and may strongly alter results due to unpredictable dose levels (44).

3. *Abnormally low levels of locomotion*. If the injection is performed too roughly, lasting pain can result and continue into the trial. This can have confounding effects on the data, especially since one of the notable phenotypes of pain in zebrafish is lethargy (exhibited by freezing and decreased locomotion). Exclude the fish from subsequent trials and discard the data, allow fish 7–10 days to heal further.

6. Anticipated Results

Our group has obtained good results with the method of i.p. injection described here. When performed correctly, behavioral and endocrine results are generally similar to those obtained using the immersion method (**Figs. 14.1 and 14.2**). We have recently conducted a pilot study to determine if adding a

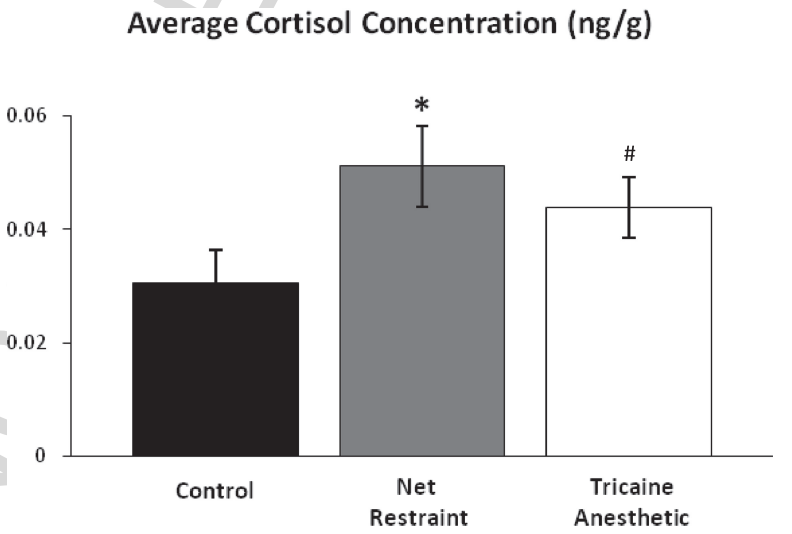

Fig. 14.2. Cortisol levels among controls (water immersion only) vs. intraperitoneal (i.p.) injection via net immobilization or using Tricaine anesthesia ($n = 13–14$). Fish receiving i.p. injection after net immobilized expressed elevated levels of cortisol vs. controls ($p<0.05$). Fish receiving i.p. injection after Tricaine anaesthetization did not exhibit significant elevations in cortisol levels (*$p<0.05$, #$p=0.05–0.1$ (trend) vs. control).

substance via i.p. injection affects behavior or cortisol levels in zebrafish. Zebrafish immobilization was achieved using two different methods. One group was trapped via net during injection, while another group was anesthetized by Tricaine for drug administration. A third (control) group remained immersed in water and did not receive i.p. injection. Overall, fish receiving i.p. injection while anesthetized by Tricaine did not show significant alterations in behavior in the 6-min novel tank test (**Fig. 14.1**), also displaying unaltered cortisol levels relative to controls (**Fig. 14.2**). However, fish immobilized via net for i.p. injection did demonstrate significant increases in cortisol vs. controls. Thus, Tricaine immobilization may be a better option for i.p. injections to avoid the confounding influences of net stress. In line with this, we have utilized this method in experiments investigating the effects of neuromodulating drugs, such as lysergic acid diethylamide (LSD). As can be seen in **Fig. 14.3**, the i.p. injection produces the results similar to those observed with the immersion method (6).

While we used i.p. injections for drug administration, other groups utilize this technique for other purposes in zebrafish, such as the injection of infectious agents to study innate immunity and bacterial pathogenesis (45, 46). As the use of biomarkers is becoming increasingly prevalent in zebrafish research, various labeling compounds can also be injected i.p., useful for the tracking of small animals and for revealing internal morphology (47–49).

While i.p. injection is not the only method of injection-based systemic drug delivery, some methods routinely used in other animals can be problematic in fish. For instance, intravenous (i.v.) injection can be difficult due to the small vessel diameter of zebrafish (50). More practical methods include intramuscular (i.m.) injections, which already were used in zebrafish studies for compounds such as salvinorin A (51), methionine enkephalin (52), the neurotoxin MPTP (53), the prostaglandin PGE2 (54), and the fluorescent tracer rhodamine dextran (55). However, this method is often not ideal as the skin seals poorly over the injection site, and large amounts of the injected substance can easily leak out (56). Likewise, intracerebral (i.c.b.) drug administration has also been applied to fish for a variety of compounds (57), but its application may be less feasible due to the animal's small size and the need for specific equipment. Subcutaneous (s.c.) injection, representing another standard practice in research involving fish, is more commonly used as an identifying marker of the animal (58, 59), but can also be applied to deliver psychotropic drug in zebrafish. Nevertheless, for most zebrafish research purposes involving treatment with pharmacological agents, i.p. injection appears to be a viable alternative to immersion.

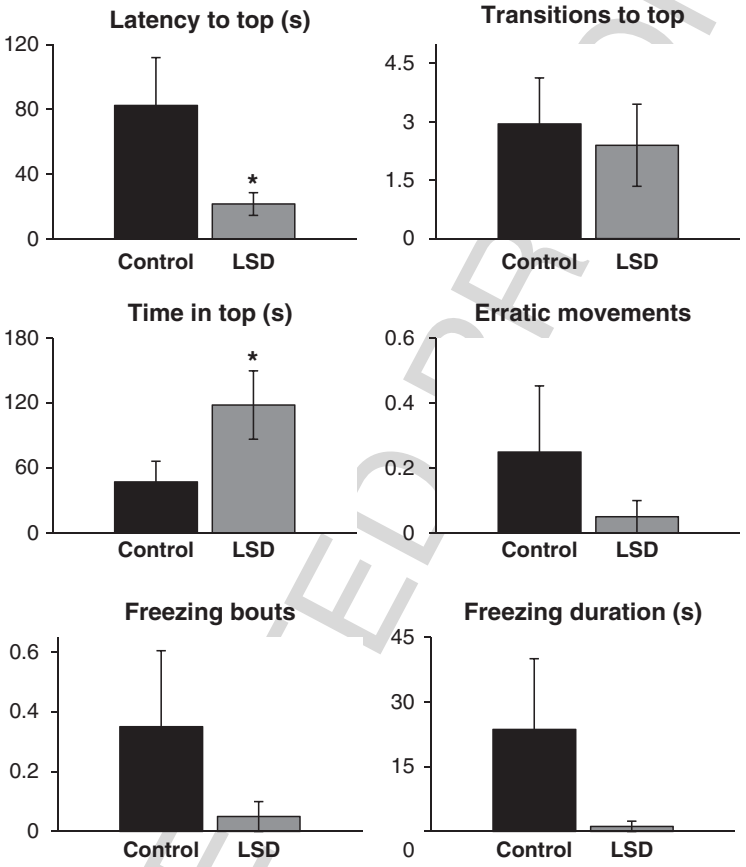

Fig. 14.3. Behavioral effects of lysergic acid diethylamide (LSD), administered to zebrafish via intraperitoneal (i.p.) injection. Control group was injected with 10 μL/fish saline solution ($n = 10$), while LSD-injected fish were injected with 10 μL of a 250 μg/L stock concentration of LSD ($n = 10$). Fish then spent 20 min in a 1 L holding beaker prior to the 6-min novel tank test. Similar to our data (6) obtained from the immersion method, LSD-injected fish had significantly lower latency to the top, more time spent in top, and tended to spend less time frozen (* $p<0.05$ vs. control).

7. Summary

Intraperitoneal injection represents a valuable technique in psychopharmacological research in zebrafish. Importantly, as new methods of behavioral quantification emerge, various effective routes of drug administration must also be available to suit the experimental design of a particular study. From this viewpoint, i.p. injection offers an easy and efficacious route of drug administration, and can complement the immersion method of drug delivery in zebrafish-based behavioral pharmacological research.

Acknowledgments

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Chapter 15

Assessing the Maximum Predictive Validity for Neuropharmacological Anxiety Screening Assays Using Zebrafish

Amanda Linker, Adam Stewart, Siddharth Gaikwad, Jonathan M. Cachat, Marco F. Elegante, Allan V. Kalueff, and Jason E. Warnick

Abstract

The development of reliable pharmacological screening assays is an important task. However, it is based upon the ability of animal models, such as the zebrafish, to demonstrate predictive validity for a specific set of drug classes. A popular assay used for this purpose is the novel tank diving paradigm, where zebrafish behavior can easily be modulated by anxiolytic or anxiogenic drug exposure. However, predictive validity may fail to provide crucial information about the model, such as comparisons of drug efficacy and the effects of drugs on varying behavioral phenotypes. This deficit is accounted for by a novel measure termed the Maximum Predictive Value (MPV), which provides an estimate of how sensitive a particular model is when assessing its potential pharmacologically. Here we provide a protocol detailing how to employ this measure to validate behavioral endpoints in the novel tank test for use in pharmacological studies in zebrafish. Similar approaches can be used to examine drug efficacy in other zebrafish-based behavioral tests.

Key words: Maximum predictive value, zebrafish, pharmacological screening, model, novel tank.

1. Introduction

In behavioral neuroscience, the use of animal models rests on the assumption that appropriate assays have been chosen to assess the desired phenotype, disease, or drug. While considerable attention has been given to the development and assessment of animal-based biobehavioral assays and simulations of neuropsychiatric disorders (1–11), little scrutiny was given to improving

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pharmacological screening assays. In determining which drug screens to employ, researchers often turn to predictive validity to assess the models' effectiveness (8–11). Predictive validity is the selectivity whereby an animal model responds to a specific class of drugs. Since these assays are primarily utilized as industrial-based tools, attainment of predictive validity is a critical necessity (10). For instance, if a screening-assay yields false negatives, researchers may unknowingly dismiss chemicals that may have therapeutic potential. Likewise, when screening assay exhibits false positives, a researcher may waste valuable resources on a substance with no potential for future development (10).

Although predictive validity is an important measure, it is dichotomous in nature (i.e., a model either does or does not possess it) (12). This represents a major shortcoming for pharmacological research, since predictive validity fails to provide the ability to differentiate the level of efficacy between drugs in a model (12). Furthermore, if multiple drug screening assays are found to possess predictive validity, this evaluative standard does not have the ability to compare the level of drug effects between these models (12). This fails to provide important information necessary for model development or selection, like statistical power, which could influence important decisions such as the number of animals to be utilized, and necessary drug dosage (12).

In an effort to move beyond the evaluative standard of predictive validity, the measure known as maximum predictive value (MPV) was developed (12, 13). This measure converts a drug's effect in a model to a standardized mean difference and allows researchers to look across multiple scores to find the largest, which provides a general estimate of how sensitive a particular model is when assessing its potential in pharmacological testing (12). This measure is a good compliment to psychopharmacology research as it accounts for several factors common to this field. First, this statistic utilizes the measures of group mean differences, which is the typical data reported in behavioral research. Second, the MPV score provides a common metric that allows the comparison of multiple models. That is, this measure provides the ability to directly compare diverse behavioral measures like the number of open arm entries in the elevated-plus maze and the amount of time spent in social contact in the social interaction test. Lastly, the measure moves beyond a simple measure of statistical significance on which predictive validity is often determined.

While statistical significance testing is an important research tool, it has major limitations that can influence the interpretation of predictive validity. For example, statistical significance can be influenced by the number of subjects used in an experiment. Thus, when a drug fails to produce a statistically significant effect, it might not reflect the model's predictive validity but in adequate sample sizes. Similarly, the experiment needs to

possess enough statistical power to produce a statistically significant effect. Therefore, a failure to produce a statistically significant effect might be due to a drug dose that is too low instead of poor predictive validity.

Again, the MPV measure allows researchers to look across multiple scores to find the largest, which provides an estimate of how sensitive a particular model is when assessing its potential in pharmacological testing (12). Due to the differences in protocols between laboratories (e.g., strain differences, drug dosage differences, vehicle differences, etc), looking across multiple studies to find the largest score keeps the findings in the context of the original study. This measure allows researchers to make critical decisions about choice of organism, drug dose, and experimental protocol (12, 13).

Assessing the MPV for a variety of pharmacological agents can reveal response patterns that would be missed by simply evaluating predictive validity (12, 13). These analyses will allow us to quantitatively assess the validity of specific behavioral endpoints, collectively revealing our model's overall validity. Moreover, the modulation of several behavioral endpoints can be used to derive a specific MPV score, such as through testing a variety of anxiolytic and anxiogenic drugs, with varying doses and durations. Additionally, the data generated using this approach, serve to identify which endpoints associated with a particular behavioral assay correlate with the highest positive MPV value (e.g., thereby indicating the drugs' ability to function as an anxiolytic or anxiogenic).

One of the most popular zebrafish behavioral paradigms is the novel tank diving test, extensively used for modeling the anxiolytic and anxiogenic properties of pharmacological agents and already comprehensively covered in this volume (*see* Chapter 1 of this book for details). Utilizing the exploratory behavior and robust endpoints exhibited by zebrafish, this assay allows for the quantification of various indices to assess a drug's overall functionality at a given dose. Here we provide a protocol that utilizes the MPV measure to assess a zebrafish model of anxiety based on the novel tank diving test, to determine which behavioral endpoints are valid constructs to test pharmacological compounds.

2. Methods and Materials

2.1. Animals and Housing

Adult zebrafish (≈50:50 male:female ratio) can be obtained from commercial distributors and tested in a standard novel tank test (refer to Chapter 1 by Cachat et al., this book for details). Room and water temperatures are maintained at 25–27°C, with illumination provided by ceiling-mounted fluorescent light tubes

on a 12-h cycle (on at 8.00, off at 20.00). All fish are experimentally naïve at the time of testing.

Apparatus: The novel tank used for this protocol is a 1.5-L trapezoidal tank (15.2 height × 27.9 top × 22.5 bottom × 7.1 width cm; Aquatic Habitats, Apopka, FL) maximally filled with aquarium-treated water. Novel tanks are to be rested on a level, stable surface and divided into two equal virtual horizontal portions, marked by a dividing line on the outside walls of the tank. The setup may also include a camera or webcam (e.g., 2.0-Megapixel, Gigaware, UK) for further video-aided analysis of recorded trials.

2.2. Maximal Predictive Validity

The behavioral data obtained from a particular experiment shows how many standard deviations apart the two groups (e.g., experimental and control cohorts) are. Data for the MPV is taken from manual and computer based observations. In the current protocol, positive MPV values indicate a drug's anxiolytic effect (reduction of anxiety-like behaviors) whereas negative values demonstrate anxiogenic effects (enhancement of an anxious state).

3. Procedure

3.1. Acclimation and Pre-treatment

Move the fish from their holding room to the experimental room for acclimation 1 h prior to testing. After acclimation, pre-treat the animals via individual immersion into a 3–4 L beaker containing the drug dissolved in ~3 L water. Drug concentration and treatment duration are determined through examination of previous literature.

3.2. Novel Tank Testing

Following pharmacological pre-treatment, zebrafish are individually placed in the novel tank. Once relocated to novel tanks, behavior should be recorded over a 6-min period manually by two trained observers and by connection to a computer. The following endpoints are recorded: number of transitions (entries) to the upper portion of the tank, time spent in the upper portion of the tank (s), number of erratic movements, number of freezing bouts, freezing duration (s), and latency to reach the upper portion of the tank (s) (14–16). Erratic movements were defined as sharp changes in direction or velocity and repeated rapid darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for 2 s or longer. Significant decreases in exploratory behavior (longer latency to reach the top, fewer entries to the top, longer freezing) or elevated erratic movements and freezing represent behavioral phenotypes indicative of high stress and anxiety (for details, *see* Cachat et al. Chapter 1, this book).

3.3. Measuring the Maximum Predictive Value of a Model

To determine the maximum predictive value (MPV), calculate the ratio of the mean difference between two groups and their pooled standard deviations as follows:

$$\text{Maximum Predictive Value} = \frac{\text{Mean}_{\text{treatment}}}{\frac{\text{Pooled Standard Deviations}}{\sqrt{2}}}.$$

$$\text{Pooled Standard Deviations} = \sqrt{\frac{(n_{\text{control}} - 1)\,\text{Variance}_{\text{control}} + (n_{\text{treatment}} - 1)\,\text{Variance}_{\text{treatment}}}{n_{\text{control}} + n_{\text{treatment}}}}.$$

Given the mathematical simplicity of this measure, our lab typically calculates MPV scores with a spreadsheet software program (e.g., Microsoft Office Excel).

4. Anticipated Results

The administration of anxiogenic and anxiolytic compounds can be expected to produce MPV values that correlate with the functionality of a drug. For example, our group has found that treatment with the anxiolytics, diazepam and fluoxetine, possess scores paralleling known drug effects. For example, in our experiments with diazepam, three of four trials resulted in significant positive MPV values for both # *of Entries to Upper Half* and *Duration In Upper Half*, with respective values of 2.268 and 2.005 for one trial, and 2.859 and 3.192 for the second, both providing interpretation as behavioral anxiolytic endpoints (**Table 15.1**). Furthermore, we have also found that fluoxetine produces a dramatic increase in MPV scores for *Duration in Upper Half*, *Average Entry Duration,* and *Latency to 1st Transition* in comparison to acute and chronic administration studies (**Table 15.1**).

However, the experimenter should also expect data of considerable complexity that warrants careful interpretation. For example, the acute administration of alarm pheromone (7 mL) can produce both anxiogenic and anxiolytic results. Our group found that zebrafish in this group demonstrate a greater # *of Erratic Movements* (MPV –1.958) and *Freezing Bouts* (MPV –1.673), as well as longer *Freezing Durations* (MPV –1.005), and an increased *Latency to the 1st Transition* (MPV –3.472). These behaviors indicate higher anxiety levels. Interestingly, the zebrafish in this group also had a higher # *of Entries to Upper*

Table 15.1
Maximum predictive validity (MPV) analyses for selected anxiolytic compounds

Experimental conditions	Endpoint	MPV score	Experimental conditions	Endpoint	MPV score
Diazepam	# of Entries to Upper Half	–0.05	Diazepam	# of Entries to Upper Half	**2.27**
Dose			*Dose*		
0.0284 mg/L	Time in Upper Half	–0.41	0.149 mg/L	Time in Upper Half	**2.01**
N			*N*		
12 control	# of Erratic Movements	0.30	12 control	# of Erratic Movements	0.16
12 experimental	Average Entry Duration	–	12 experimental	Average Entry Duration	–
	# of Freezing Bouts	–0.58		# of Freezing Bouts	–0.90
	Freezing Duration	–0.58		Freezing Duration	–0.88
	Latency to 1st Transition	–		Latency to 1st Transition	–
Diazepam	# of Entries to Upper Half	**2.86**	Diazepam	# of Entries to Upper Half	0.86
Study I			Study II		
Dose			*Dose*		
3.6 mg/0.05 l	Time in Upper Half	**3.19**	3.6 mg/0.05 l	Time in Upper Half	0.65
5 min exposure			5 min exposure		
N			*N*		
10 control	# of Erratic Movements	0.32	10 control	# of Erratic Movements	–0.66
10 experimental	Average Entry Duration	–	11 experimental	Average Entry Duration	–
	# of Freezing Bouts	–0.95		# of Freezing Bouts	–1.43
	Freezing Duration	–0.81		Freezing Duration	–1.09
	Latency to 1st Transition	–		Latency to 1st Transition	–

Table 15.1 (continued)

Experimental conditions	Endpoint	MPV score	Experimental conditions	Endpoint	MPV score
Fluoxetine (Acute)	# of Entries to Upper Half	0.28	Fluoxetine (Chronic) 3 weeks	# of Entries to Upper Half	–0.54
Dose			*Dose*		
100 μg/L	Time in Upper Half	0.10	100 μg/L	Time in Upper Half	**–9.13**
N			*N*		
16 control	# of Erratic Movements	–0.11	5 control	# of Erratic Movements	0.84
14 experimental	Average Entry Duration	–0.49	4 experimental	Average Entry Duration	**–3.04**
	# of Freezing Bouts	0.80		# of Freezing Bouts	1.37
	Freezing Duration	0.83		Freezing Duration	1.08
	Latency to 1st Transition	0.23		Latency to 1st Transition	**3.28**

Bold numbers indicate statistically significant results ($P < 0.05$).

Table 15.2
Maximum predictive validity (MPV) analyses for selected anxiogenic manipulations

Experimental conditions	Endpoint	MPV score	Experimental conditions	Endpoint	MPV score
Alarm Pheromone	# of Entries to Upper Half	0.06	Caffeine	# of Entries to Upper Half	1.00
Dose			*Dose*		
200 mL undiluted	Time in Upper Half	0.81	100 mg/L 15 min exposure	Time in Upper Half	0.95
N			*N*		
10 control	# of Erratic Movements	–1.34	21 experimental	# of Erratic Movements	–0.85
10 experimental	Average Entry Duration	0.31	21 experimental	Average Entry Duration	0.77
	# of Freezing Bouts	**–14.43**		# of Freezing Bouts	–0.30
	Freezing Duration	0.04		Freezing Duration	–0.27
	Latency to 1st Transition	0.06		Latency to 1st Transition	–

Half (MPV 3.559) and spent a great amount of *Time in the Upper Half* (MPV 2.381). Furthermore, we have found that subjects receiving 200 mL of undiluted alarm pheromone had positive MPV scores in all behavior parameters according to the pilot data. However, in another study at this dose, zebrafish had an MPV of –14.425 in # *of Freezing Bouts*, indicating an anxiogenic effect (**Table 15.2**). Likewise, the acute administration of caffeine also appears to induce anxiogenic symptoms in our zebrafish. For example, subjects treated with 100 mg/L of caffeine displayed an increase in # *of Erratic Movements* and *Freezing Bouts* and experienced longer episodes of freezing behavior (**Table 15.2**).

5. Summary

Using the MPV measure can be a beneficial tool for the development and characterization of new animal models for behavioral pharmacology research. In this protocol, the MPV measure allowed our laboratory to analyze multiple behavioral measures to assess drug efficacy and treatment reliability. This also allows for the assessment of validity while also enabling fine-grained analysis not addressed by the dichotomous measure of predictive validity (*see* above) (12). For example, a promising measure resulting from our alarm pheromone trials is the *Freeze Duration* measure, which produced an expected anxiogenic response. This suggests a potential importance of employing this specific behavioral endpoint when analyzing anxiogenic compounds.

The strength of the MPV as an analytical tool is most profound when observing our diazepam results. Diazepam would be expected to produce effects associated with eliminating the fear response like more frequent trips and spending more time in the upper half of the tank. These behaviors would likely be anxiety-provoking to zebrafish in their native environment due to the risk of predators near the water's surface. In our studies, the MPV value calculated for two of the three trials give positive values associated with # *of Entries to Upper Half* and *Duration in Upper Half* as valid behavioral endpoints in assessing diazepam as an anxiolytic. It is important to note that all trials except for the lowest dosage yielded positive values for these two endpoints. This represents consistency and reliability for these measures in regards to accurately representing diazepam as an anxiolytic compound. Analyzing MPV values for specific endpoints across different trials can help elucidate information such as the most effective dose, as seen by the increasing MPV values when increasing the dosage from 0.149 mg/L to the 3.6 mg/L. Collectively, this provided further evidence that the MPV measure can allow a researcher to

make precise decisions about drug doses for specific compounds that goes beyond the measure of predictive validity (12, 13).

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Chapter 16

Deconstructing Adult Zebrafish Behavior with Swim Trace Visualizations

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Abstract

Three-dimensional reconstruction is a method of qualifying the behavioral activity of several animals including mice, rats, and zebrafish. This method allows for measuring behavioral endpoint data on two types of tracking planes (temporal and spatial). Temporal tracking measures the activity of a subject across time while spatial tracking measures the activity of a subject in a specific area of the experimental arena as such. Data representation over 3D visual trajectory reconstruction is a robustly advantageous method of behavioral phenotyping. Digital video-tracking and analysis eliminates the inaccuracies of manual tracking and allows for improved investigation of behavioral activity at specific points in time or specific areas of the tracking arena.

Key words: Zebrafish, behavioral endpoints, novel tank test, video-tracking technology, affective behaviors, 3D reconstruction.

1. Introduction

The use of video-tracking software in neuroscience research has markedly advanced neurobehavioral phenotyping by permitting rapid, more objective quantification of the animal activity. Video-tracking programs standardize and automate behavioral endpoints, promoting reproducibility of phenotypic studies and allowing for multiple endpoints to be recorded at once (1) (**Figs. 16.1, and 16.2**).

Three-dimensional (3D) trajectory reconstruction is a method of behavioral analysis that has been performed in multiple animal models ranging across insects, rodents, and primates

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A

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Header Li	33											
2	Experim	3-17-10\NicotineSideTop											
3	System Independent Variable												
4	Trial nam	Trial 7											
5	Trial ID	6											
6	Arena na	Arena 1											
7	Arena inc	0											
8	Subject r	Subject 1											
9	Object in	0											
10	Arena se	Arena Settings 1											
11	Detection	Detection Settings 1											
12	Trial cont	Trial Control Settings 1											
13	Start time	20:52:5											
14	Trial Dura	+ 0:06:01.1											
15	Recordin	+ 0:00:01.0											
16	Recordin	+ 0:05:00.1											
17	Track	Track file\0006a0000o0000_0001.trk											
18	Tracking source												
19	Video file Z:\Video\hub\Zebrafish\(3.17.10)Nicotine~10mgL@5min~NT~3Dh(3-17~10)5min@10mgL~Nicotine~Side~C7.avi												
20	Video sta	20:46:0											
21	Trial status												
22	Acquisitic	Acquired											
23	Track sta	Acquired											
24	Video file	External											
25	Sync stat	Not available											
26	Referenc	+ 0:06:01.1											
27	Referenc	20:52:5											
28	Sof file												
29	User-defined Independent Variable												
30	Fish Grou	Control											
31	Fish ID	C7											
32													
33	Trial time	Recordi	X cente	Y cente	X nose	Y nose	X tail	Y tail	Area	Areach	Elongat	Directio	Distanc
34	1.033	0	-0.048	-0.057	-0.037	-0.06	-0.057	-0.057	0.0001	0	0.9178	-15.07	-
35	1.066	0.033	-0.048	-0.057	-0.037	-0.06	-0.057	-0.057	0.0001	0	0.9178	-15.07	5E-06

B

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Time	X	Y	Area	Areacha	Elongatic	Direction	Distance	Velocity	In zone	TurnAngl	TurningR	TurnBias
2	0	-0.048	-0.057	0.0001	0	0.9178	-15.0662	5.5E-06	0.00016	0	43.3179	1479.55	1479.55
3	0.033	-0.048	-0.057	0.0001	0	0.9178	-15.0662	5.5E-06	0.00016	0	44.9735	1349.22	1349.22
4	0.066	-0.048	-0.057	0.0001	0	0.9178	-15.0662	3.7E-06	0.00011	0	43.3179	1479.55	1479.55
5	0.099	-0.048	-0.057	0.0001	0	0.9178	-15.0662	4.7E-06	0.00014	0	44.9735	1349.22	1349.22
6	0.133	-0.048	-0.057	0.0001	0	0.9178	-15.0662	6.4E-06	0.00019	0	19.6196	588.576	588.576
7	0.166	-0.048	-0.057	0.0001	0	0.9178	-21.0724	0E+00	0.00024	0	19.6627	301.003	301.003
8	0.199	-0.048	-0.057	0.0001	0	0.9178	-15.0662	9.2E-06	0.00028	0	6.37719	191.318	191.318
9	0.233	-0.048	-0.057	0.0001	0	0.9178	-15.0662	1E-05	0.00031	0	4.56831	137.046	137.046
10	0.266	-0.048	-0.057	0.0001	5.3E-07	0.91706	-18.1918	1.1E-05	0.00033	0	3.51366	105.411	105.411
11	0.299	-0.048	-0.057	0.0001	3.6E-07	0.91682	-24.9461	1.2E-05	0.00035	0	2.33206	84.9626	84.9626
12	0.333	-0.048	-0.057	0.0001	0	0.91682	-16.5131	1.2E-05	0.00037	0	2.37666	71.2985	71.2985
13	0.366	-0.048	-0.057	0.0001	0	0.91682	-16.5131	1.8E-05	0.00054	0	3.35897	91.7693	91.7693
14	0.399	-0.048	-0.057	0.0001	0	0.91682	-16.5131	1.9E-05	0.00057	0	4.17834	125.351	125.351
15	0.433	-0.048	-0.057	0.0001	1.2E-05	0.91397	-16.5131	1.9E-05	0.00056	0	2.8822	84.067	84.067
16	0.466	-0.048	-0.057	0.0001	0	0.91397	-16.5131	1.7E-05	0.00051	0	1.34737	31.4206	31.4206
17	0.499	-0.048	-0.057	0.0001	0	0.91397	-16.5131	1.5E-05	0.00044	0	0.09841	2.95245	2.95245
18	0.533	-0.048	-0.057	0.0001	0	0.91397	-16.5131	1.2E-05	0.00036	0	3.23406	97.0227	-97.0227
19	0.566	-0.048	-0.057	0.0001	0	0.91397	-16.5131	9.2E-06	0.00029	0	2.49715	74.9163	74.9163
20	0.599	-0.048	-0.057	0.0001	0	0.91397	-16.5131	5.7E-06	0.00017	0	14.8573	445.723	-445.723
21	0.633	-0.048	-0.057	0.0001	0	0.91397	-16.5131	3E-06	8.9E-05	0	19.2284	576.858	576.858
22	0.666	-0.048	-0.057	0.0001	0	0.91397	-16.5131	2.2E-06	6.7E-05	0	21.4606	643.805	643.805
23	0.699	-0.048	-0.057	0.0001	0	0.91397	-16.5131	2E-06	6E-05	0	104.118	3123.56	3123.56
24	0.733	-0.048	-0.057	0.0001	0	0.91397	-16.5131	5.4E-06	0.00016	0	15.0712	452.14	452.14
25	0.766	-0.048	-0.057	0.0001	0	0.91397	-24.9461	9.2E-06	0.00028	0	35.3001	1059.01	-1059.01
26	0.799	-0.048	-0.057	0.0001	0	0.91397	-16.5131	7.5E-06	0.00022	0	16.1137	483.401	-483.401
27	0.833	-0.048	-0.057	0.0001	0	0.91397	-16.5131	8.5E-06	0.00025	0	1.58186	47.4562	47.4562
28	0.866	-0.048	-0.057	0.0001	3.6E-07	0.91351	-11.1343	9.3E-06	0.00028	0	0.34746	10.4238	10.4238
29	0.899	-0.048	-0.057	0.0001	0	0.91351	-5.63198	1.1E-05	0.00034	0	6.22508	186.749	186.749
30	0.933	-0.048	-0.057	0.0001	1.3E-05	0.90786	-3.88821	1.1E-05	0.00032	0	3.39664	119.9	-119.9
31	0.966	-0.048	-0.057	0.0001	0	0.90786	-3.88821	5.2E-06	0.00028	0	5.52627	165.79	-165.79
32	0.999	-0.048	-0.057	0.0001	0	0.90786	-3.88821	7.6E-06	0.00023	0	7.55982	226.79	-226.79
33	1.033	-0.048	-0.057	0.0001	0	0.90786	-9.40451	5.9E-06	0.00018	0	11.0943	332.831	-332.831
34	1.066	-0.048	-0.057	0.0001	0	0.90786	-9.40451	4.5E-06	0.00013	0	14.7203	441.614	-441.614
35	1.099	-0.048	-0.057	0.0001	0	0.90786	-3.88821	6.5E-06	0.00019	0	155.627	4668.85	-4668.85

Fig. 16.1. An example of a track data sheet exported by Noldus EthoVision XT7. **a** – Initial, unprocessed track data obtained from the software. **b** – Formatted, processed track data (after removing identification information, removing spaces from column headers and null values) ready for import into RapidMiner for 3D visualization.

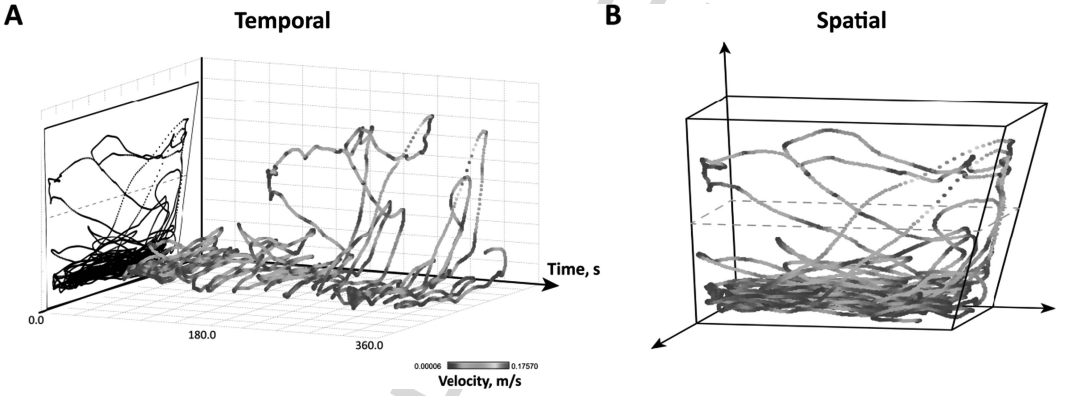

Fig. 16.2. Swim Path 3D Reconstructions. **a** – Temporal swim trace reconstruction of wild-type control fish in standard 6-min novel tank test. **b** – Complete (spatial) reconstruction of same wild-type control fish. Note that time is used as the third axis for temporal reconstructions (**a**) and Cartesian coordinates form the framework for spatial reconstructions (**b**).

(2–4). Video analysis has also been used in zebrafish model to determine specific behavioral endpoints such as velocity, total distance traveled, angular characteristics, and mobility (5). However, previous studies using 2D video analysis have not been able to fully characterize the behavioral activity of zebrafish, suggesting that some behaviors must be analyzed using 3D model-based tracking when analyzing body kinematics (6). A recent study recorded and analyzed individual fish behavior using video-tracking software in a 2D-coordinate plane, and noting errors when two fish crossed paths (7).

Motion-based information in 3D video capture can be obtained through video-tracking programs that occlude human error and inter/intra-rater reliability, and consequently avoid subjective misinterpretation (8). Collectively, this indicates the

importance of 3D-based behavioral analyses in neuroscience research as a more precise method that characterizes zebrafish behavior more comprehensively (e.g., (9)).

Three-dimensional trajectory reconstruction described here involves two major approaches, including spatial and temporal modeling. Temporal reconstructions (**Fig. 16.2a**) visualize zebrafish activity across experimental time, while spatial reconstructions map behavioral changes in real spatial regions of the arena (**Fig. 16.2b**). The tracks produced by plotting the temporal activity allows for visualization of the behavior of the zebrafish at specific points in time. The tracks produced by measuring the spatial activity allows for visualization of the behavior of the zebrafish in specific areas of the arena.

The goal of this chapter is to introduce 3D trajectory reconstruction as a method of tracking zebrafish behavior and to explain how the data collected from the 3D reconstruction can be used to complement the measurement of specific zebrafish behaviors.

2. Materials

Adult zebrafish (~50:50 male:female) can be obtained from a commercial distributor or from the growing availability of genetic mutants at the Zebrafish Information Network (ZFIN.org). Generally, all fish should be given at least 10 days to acclimate to the laboratory environment and be experimentally naïve, although these conditions may vary with the nature of each investigation. Fish can be housed in groups of 20–30 fish in a 40-L tank with water and room temperature maintained at 25–27°C. Illumination can be provided by ceiling fluorescent light bulbs on a 12-h cycle (06:00–18:00 h).

3. Experimental Setup

There are a number of behavioral paradigms for adult zebrafish, with unique experimental specifications thoroughly described within each respective chapter of this volume. For video-tracking and swim track analysis, several universal precautions should be considered. To achieve a precise subject detection, sharp contrast between the subject(s) and the arena background is required. For example, laminated white sheets of paper can be placed behind or below the behavioral apparatus. Reflections from ambient lighting on both the experimental tank and the water line should also be

minimized. Care should be taken to ensure that the camera position does not shift between experimental trials. Notably, video recording does not require premium resolution camera equipment. Our group has found that lower-resolution (320 × 240 or 640 × 480), high frame rate (30 fps) videos are preferable due to smaller file size and subject-to-background pixel representation.

3.1. Time Requirement

Time requirement varies according to behavioral paradigm and track data processing requirements. A typical experiment involving 10 control and 10 experimental fish will require ~2.5 h for capturing the videos and ~2.5 h to analyze the videos. If the laboratory has the ability to record and analyze videos "live" (which requires a video-digitizing system), this time can be reduced to only 2.5 h. Exporting and formatting the data, as well as visual reconstruction of traces in RapidMiner, will require additional 2–3 h.

3.2. General Procedure

There are several available video-tracking software technologies including both commercial and open source packages. Our group has successfully used both LocoScan (CleverSys, Inc. Reston, VA) and EthoVision XT7 (Noldus Information Technologies, The Netherlands) for adult zebrafish behavioral analysis. This chapter will focus on Ethovision XT7, although the protocol can easily be applied to any video-tracking software, provided time-series and spatial coordinate data can be exported.

4. Video and Track Analysis

Since the procedure for video-aided analysis of zebrafish behavior is detailed in another chapter (Chapter 1) of this volume, this protocol will focus on supplementary procedures pertinent for swim track visualization.

For each experiment, an arena and respective zones are established over the experimental tank in order to focus detection at the location of swimming activity. While calibrating each arena, specific attention should be given to the placement of the calibration axes, which specify *x*-, *y*-coordinate values. By default, EthoVision XT7 places the origin of the calibration axes at the center of the image. However, for different behavioral paradigms, it may be beneficial to customize the origin location. For example, in a standard novel tank test the origin axis is placed along the dividing middle line, denoting the center of the tank as (0, 0). Although customizing the calibration axes is not required, knowing the origin location relative to the testing arena is critical for visual reconstructions.

After all videos are acquired for an experiment, tracks should be smoothed (across 10 samples) and examined for each trial for

any abnormalities (i.e., bad detection or wrong arena). Smoothing is required to eliminate "noise" or small bodily movements that are irrelevant to the data collection and could lead to an overestimation of, for example, the total distance traveled. In the event of major issues, it is recommended to reacquire afflicted videos with properly revised detection settings. Ethovision XT7 includes a basic track visualization feature in which 2D swim paths are plotted within the arena and can be saved as an image file for future reference. For details on troubleshooting, refer to Notes 1–4.

Following a full examination of the behavioral analysis export, tracks need to be interpolated for each trial in order to complete track data across the trial duration. This step replaces missing spatial coordinates by a linear interpolation of the nearest neighbor detection points or the previous and most recent valid detection coordinates. In EthoVision XT7, interpolation is performed within the track editor. At this point, rogue track points (i.e., brief jumps outside of the arena) should be removed prior to interpolation.

4.1. Track Processing

1. Export swim path data as an Excel file from EthoVision XT7 by selecting "Export", "Raw Data…" and then checking "Track & dependent variables" option. Track data exported from EthoVision XT7 provides spatial coordinates and endpoint values for each trial across a time scale broken down into fractions of a second. Based on the frame rate of the acquired video, values are typically provided at 0.033 or 0.024 of every second.
2. In Excel, open each export file and save a copy to a separate folder, naming the file based on the subject's ID (i.e., Control1). This is to ensure that the original export files are not manipulated and each subject's swim path can be identified in the future. Once a copy is saved with necessary identification information in the file name, delete all cells above the raw data positioning the column headers (i.e., Trial time, Recording time, X center…) in the first row (**Fig. 16.1**).
3. Rename each column header, or dependent variables, to remove all spaces. For example, "Recording time" to "RecordingTime" or "X center" to "X".
4. In order for tracks to be properly imported into RapidMiner, there cannot be null ("-") values in the first time point for each variable. If found, null values should be replaced by the first valid data point or the entire time point(s) (i.e., entire row) can be deleted. This corrective procedure should be limited to the first 0.5 s. All remaining null values "-" need to be replaced as blank cells. To change all "-" to blank,

perform a "find and replace all" procedure with the "match entire cell contents" option checked.

5. Depending on research interests, specific dependent variables can be removed by selecting and deleting the entire column. Additionally, discrete dependent variables (i.e., Movement, Elongation), exported as binary values (0 = false, 1 = true), can be merged with custom "Find and Replace" as well as "concatenate" templates or macros in Excel.
6. For complete 3D reconstructions, raw track data from both side and top views should be processed and corrected separately. Following, the spatial coordinates from the top view can be merged into the side view excel sheet as "Xtop" and "Ytop", respectively, using the recording time, trial time, and/or unique video time stamps (i.e., the fish being placed into the tank) for proper synchronization. Note that larger arenas (i.e., open field tests) may require more advanced calibration procedures to synchronize the spatiotemporal data of two videos.
7. After initial processing steps, each track file is subsequently saved as a comma separated value (CSV) file.

4.2. RapidMiner

1. Obtained track files are then imported into RapidMiner 5.0 Community Edition, an open-source data analysis and mining system (Rapid-I GmbH, Germany). The corrected track files are imported as separate CSV example sources within a single process based on the goal(s) of the reconstruction process. This permits each track to be explored in a variety of plots after the process is run. Each column is designated as either a real or integer value type based on its contents and no special attributes are assigned. For details on troubleshooting, refer to Notes 5–6.
2. Temporal 3D reconstructions (**Fig. 16.2a**) are created with a Scatter 3D Color plotter, in which the X center, Trial time, and Y center are plotted on the *x*-, *y*-, and *z*-axes, respectively. Complete (spatial) 3D reconstructions (**Fig. 16.2b**) are also produced with a Scatter 3D Color plotter, but X center (side), X center (top), and Y center (side) are plotted on the *x*-, *y*-, and *z*-axes, respectively. Subsequently, select dependent variables (i.e., velocity, elongation, direction) are actively cycled across the reconstructed trace as the Color attribute. In order to compare across fish and/or experimental cohorts, the bounds of each axis are standardized. Images and videos of these reconstructions are exported or captured using screen capture software (Debut Video Capture, NCH Software, Australia). For details on troubleshooting, refer to Notes 7.

5. Anticipated/ Typical Results

Swim trace visualizations are beneficial to all aspects of behavioral research. In the novel tank test, 3D trajectory visualization renderings allow for color-based separation of microscopic behavioral activity across a real-time depiction of fish movement (**Figs. 16.3, and 16.4**). Such an interactive, investigative environment offers a significant advantage when comparing multiple behavioral endpoints. Typically, behavior between control and experimental cohorts are assessed by comparing bar, column, or line graphs of each quantified endpoint. As the number of valuable behavioral endpoints increase, these methods of data representation are less practical because comparing 20+ bar graphs between or across experimental trials is incomprehensible.

With 3D reconstructions, we have the capacity to both manipulate resolution and orientation of the real-time virtual movement model and view geometric trends occurring over time. With advances in computational neuroscience, 1D graph and 2D trace modeling of behavioral parameters have become methods of extrapolating the same information conveyed in a 3D model, except to a higher volume. Printouts of 3D Model traces can be easily viewed side by side to compare any physically defined trend, such as bouts of freezing and erratic movement (**Figs. 16.1–16.3**). Software-mediated manual recording of behaviors can be compared for reliability to automated software-run recordings. Furthermore, each reconstruction can easily be saved in a file for later review.

Finally, by cycling various behavioral endpoints across the same swim track reconstruction, we can compare manually registered activity with automated endpoints (**Fig. 16.3**). Performing this procedure across multiple endpoints and several experimental challenges will improve customization settings in video tracking software. Eventually, such comparisons will advance automated event detection to allow software to register endpoints once previously limited to manual quantification.

6. Notes

1. *Video-tracking software:* For issues involving arena settings, subject detection and/or behavioral variables, please consult the troubleshooting **Section 11** of the video-tracking Chapter 1 by Cachat et al. in this book.

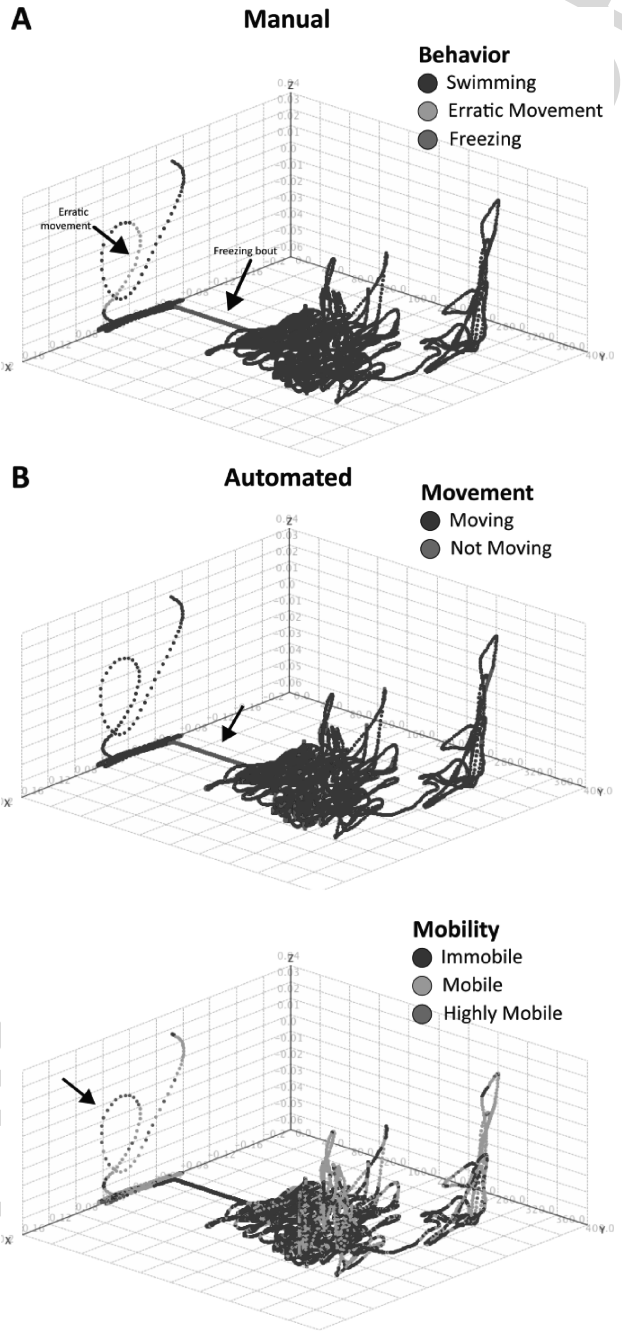

Fig. 16.3. Comparison of manual and automated behavioral endpoints for temporal reconstructions of a typical wild-type control fish tested in a standard 6-min novel tank test. Manual behavior (**a**) was registered during video acquisition using EthoVision XT7. Automated quantification of "Movement" was applied to behavioral endpoints (note correlation of *not moving* here with *freezing* in panel **a**), and "Mobility" (note correlation of highly mobile here with erratic movement in Panel **a**).

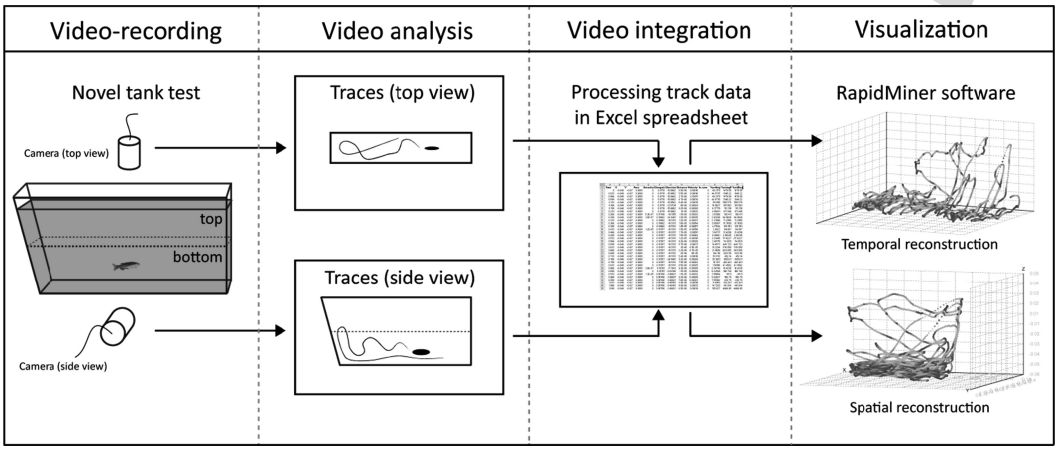

Fig. 16.4. A summary of the swim path reconstruction process.

2. *Trial ID and Subject ID lost:* After removing the identification information from the raw track data export, if the new file is not named with a unique subject ID, the swim path cannot easily be identified as a specific fish. By saving the processed track data as a new file, in a separate folder, the original export file can always be consulted for identification information. As a last resort, the original video analysis experiment can be reviewed in EthoVision to correlate Trial IDs to specific Subject IDs.
3. *Swim path points outside of arena:* Irregularities in the swim path should be noticed and corrected within the EthoVision XT7 track editor. For example, EthoVision will occasionally detect background spots as the subject causing the swim track to jump outside of the experimental tank. Such points should be removed by smoothing the track, but can be manually removed within the track editor prior to interpolation. However, sound judgment should be practiced during such manipulation. If these rogue points are frequent across all experimental trials, it is recommended to reanalyze the videos with revised detection settings.
4. *Swim path points fixed or congregated abnormally:* If there is an unusual point within the arena where the swim track repeatedly "jumps" to or centers around, this is typically the result of a reflection in the video. Especially in top view videos, video-tracking software can easily confuse the movement of a surface reflection for that of the subject. In most cases, this requires rerecording the videos after all reflections have been eliminated or minimized.
5. *RapidMiner shifting column headers:* Open the affected CSV file(s) in Excel and check that each column header is labeled without spaces.

6. *RapidMiner incorrectly importing CSV data:* Generally, errors importing data into RapidMiner occur because the CSV file is not formatted correctly. Additionally, make sure that the first value of each variable is not null and contains a real number, whereas all remaining null "–" values have been replaced as empty cells. All columns must also contain the same number of rows. In a few cases, we have found that the last row of some dependent variables is reported as null. This can be corrected by copying the last valid value or removing the time point entirely for all endpoints. If these corrective measures have been taken and import issues remain, it is possible that a single dependent variable is causing a nonspecific import issues. Consider limiting the track file to Time, X and Y coordinate values, resaving the CSV file and performing the import procedure. Provided the import processes correctly, dependent variables can then be included one at a time as necessary.
7. *Swim path visualization appears abnormal:* As mentioned previously, noting the placement of the calibration axes when establishing the arena settings is critical. Initial 3D scatter plots can appear incorrect because the RapidMiner automatically chooses the max/min range for each axis of the scatter plot. Standardizing the calibration axes during video analysis and manipulating the scatter plot axis ranges will improve the swim path visualization.

7. Conclusion

Three-dimensional trajectory reconstruction offers important opportunities to improve behavioral phenotyping of zebrafish. The data acquired through 3D reconstruction (**Figs. 16.1–16.4**) can provide extensions to pinpointing measurements of specific behavioral endpoints that are not measurable by simple video-tracking (i.e., erratic movements). Data collected for average velocity indicate a correlation that can be used in the video-tracking software to set range of predetermined values where a specific behavioral endpoint will occur. Finally, 3D trajectory reconstruction also allows for manipulation within the tracking software to better characterize the behavior of the zebrafish.

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Index

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