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Measuring Behavioral Individuality in the Acoustic Startle Behavior in Zebrafish

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[Abstract] The objective of this protocol is to provide a detailed description for the construction and use of a behavioral apparatus, the zBox, for high-throughput behavioral measurements in larval zebrafish (*Danio rerio*). The zBox is used to measure behavior in multiple individuals simultaneously. Individual fish are housed in wells of multi-well plates and receive acoustic/vibration stimuli with simultaneous recording of behavior. Automated analysis of behavioral movies is performed with MATLAB scripts. This protocol was adapted from two of our previously published papers (Levitz *et al.*, 2013; Pantoja *et al.*, 2016). The zBox provides an easy to setup flexible platform for behavioral experiments in zebrafish larvae.
Keywords: Zebrafish, Behavior, Individuality, Neuromodulation, High-throughput, Startle, Larvae

[Background] Behavioral differences between individuals in a population are ubiquitous and likely play a role in adaptation to varying selective pressures during evolution. However, variation in behavior among individuals is often ignored when the quantitation of the behavior of groups is presented as means and associated dispersions. In this protocol, we describe an approach to characterize individuality in the habituation of the acoustic startle behavior at the population level in zebrafish (*Danio rerio*) larvae. Our approach and set-up can be easily adapted to studies of individuality in other zebrafish larval behaviors.

Materials and Reagents

1. Petri dishes
2. Pipette tip
3. 48-well multi-well plate
4. Zebrafish embryos
5. Instant ocean sea salt mix (Spectrum Brands, catalog number: SS15-10)
6. DI H_2O

7. E3 medium (see Recipes)

Equipment

1. P1000 PIPETMAN pipettor
2. Stir plate
3. zBox parts list (Table 1)

Table 1. ZBox parts list

Manufacturer/Item description	Part number	Quantity
Newegg - Computer		
Minimum requirements: PC, 8 GB RAM, 2 GHz processor		1
PCIe Firewire 800 card	9SIA24G28M5974	1
National Instruments (DAQ)		
NI PCI-6229	779068-01	1
RC68-68 Cable (1 m)	192061-01	2
CB-68LP - Unshielded	777145-01	2
McMaster Carr (Structural external)		
80/20 1"x1" framing 96"	47065T123	4
80/20 corners	47065T177	20
80/20 nuts 80x	47065T147	12
Black PVC, 48"x48"x.	84775K942	1
236" (back) Black PVC 36"x48"x.236	84775K742	3
sides and top Super Adhesive Hook	94985K614	10ft
Loop	9489K874	10ft
Loctite Black Silicone sealant	74945A81	1
Black gaffer's tape	7612A94	1
Right angle 80/20	47065T223	2
80/20 fastener	47065T139	2
Acrylic cement	7528A13	1
PTFE tape	4591K11	1
General Purpose Epoxy	Permatex 84101	1
Nylon 6/6 Thumb Screw	96295A336	1
Thorlabs (Structural internal)		
Breadboard 24" x 36"	MB2436	1
XT66 Rail	XT66-500	1
XT66 Dovetail	XT66DP-500	1
XT66 Dovetail clamp 40 mm	XT66C2	2
XT66 right angle cross piece	XT66CB	1
XT66 base plate	XT66P1	1
XT66 pivot platform	XT66RC	2
Plate holder	FP01	2
10" mounting post	P10	1
Pedestal Base adapter	PB4	1
Clamping fork	PF175	1
Post mounting clamp	C1501	1
Blackout fabric	BK5	1
Right angle plate	AP90	1
Edmund Optics		
101 x 127 mm, 45 Degree AOI, Hot Mirror	43-958	2

Table 1 Continued

Pike F-245 2/3" CCD camera	59-221	1
Pike Tripod adapter	59-227	
Plastic IR filter	43-949	1
Navitar		
50MM F/2.8 TELECENTRIC LENS	TC-5028	1
Unibrain (1394store.com)		
1m (15 ft) IEEE-1394b 9p to 9p screw lock cable		1
Digi-Key Electronics		
100Kohm resistor	CMF100KHFCT-ND	4
2-pin recipient	A99613-ND	10
2-pin socket	A99620-ND	20
2-pin header	A30770-ND	10
Yellow LEDs	C503B-AAN-CY0B0251-ND	48
IR LEDs	475-2919-ND	48
White LEDs	C503C-WAS-CBADA151-ND	48
Blue LEDs	C503B-BAN-CY0C0461-ND	48
1ohm resistor	P1.0W-2BK-ND	3
270 ohm resistor	PPC270W-1CT-ND	16
390 ohm resistor	PPC390W-1CT-ND	6
Diode	1N914B-ND	2
12 V power supply	EPS377-ND	1
24 V power supply	EPS357-ND	1
LEDsupply		
BuckPuck DC LED Drivers	0302x-D-x-xxxx	1
Machine shop (plastics)		
Custom plastic fabrication		1
Whatman (GE) plates		
Multi-well plate of desired well number		
Visaton (speakers)	8006	2
Pyle Audio (amplifier)	PyleHome PCA1	1
60 Watt incandescent bulb		1
Simple designs home (lamp)	LD1003-WHT	1

Notes: Assembly of zBox (see Figures 1-7):

a. *Use epoxy to adhere the two side walls into the grooves on the back panel (Figure 2).*

b. *The screw holes in the LED platforms need to be drilled for 4-40 threads (Figure 3).*

c. *Use the plastic thumbscrews to mount the 2 LED platforms (one for the imaging IR LEDs and one for LEDs for use in optogenetic experiments) to the slots in the side walls (Figure 4).*

d. *LED arrays can be wired in 8 x 6 or 12 x 10 arrays depending on the desired amount of light. Due to perspective, the IR imaging LEDs are best arranged so that the arrays extend to the sides of the LED platform. Thus, given the distance from the plate, the IR LEDs can evenly light the extent of the multiwell plate.*

e. The BuckPack LED driver can be wired to power the LED arrays and be switched using TTL pulses from the DAQ

f. The multiwell plate platform should be able to slide into the grooves on the side walls.

g. Screw speakers to side of multiwell platform (Figures 4-6).

h. Use diffuser paper to assure that IR light is evenly distributed throughout wells.

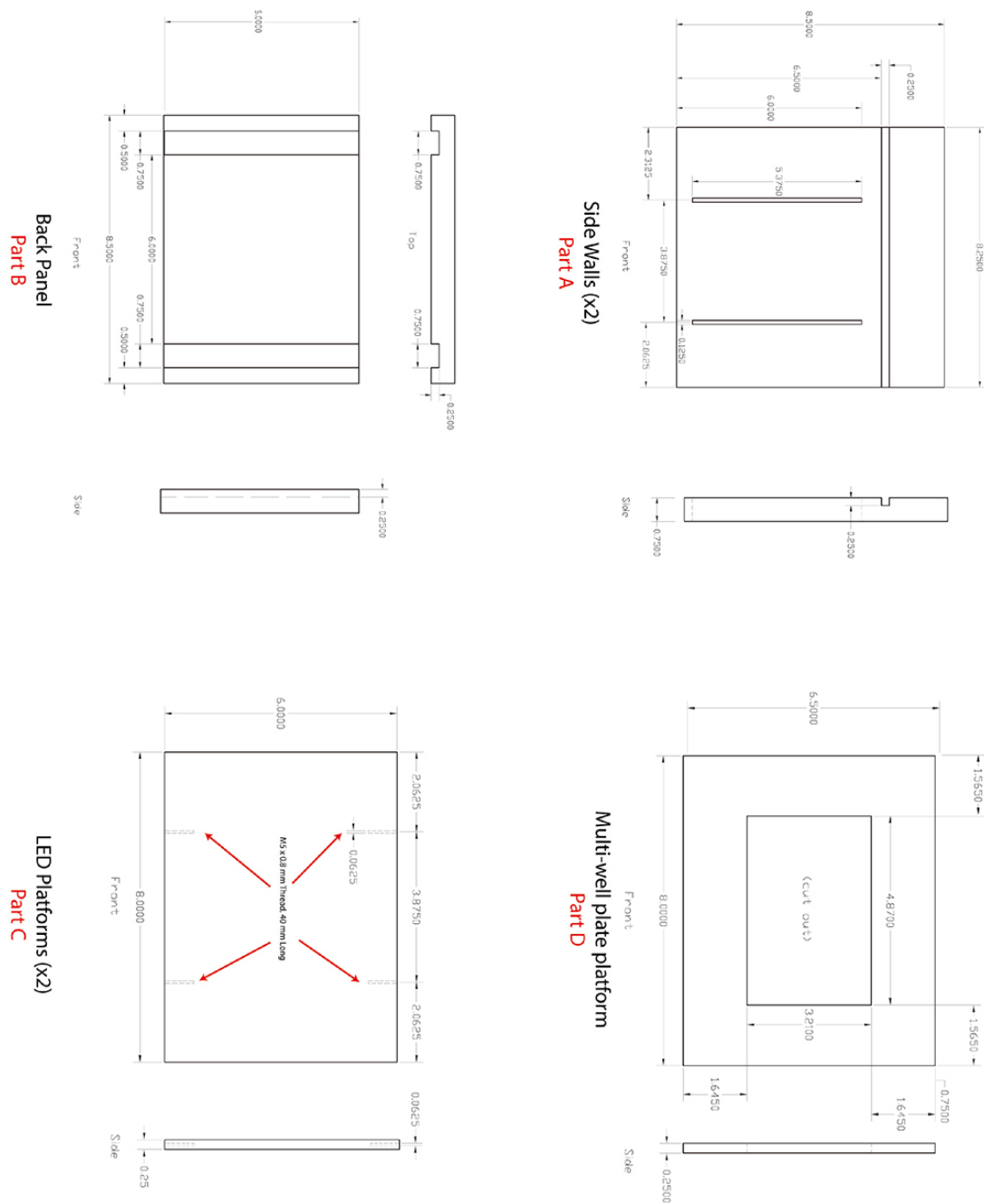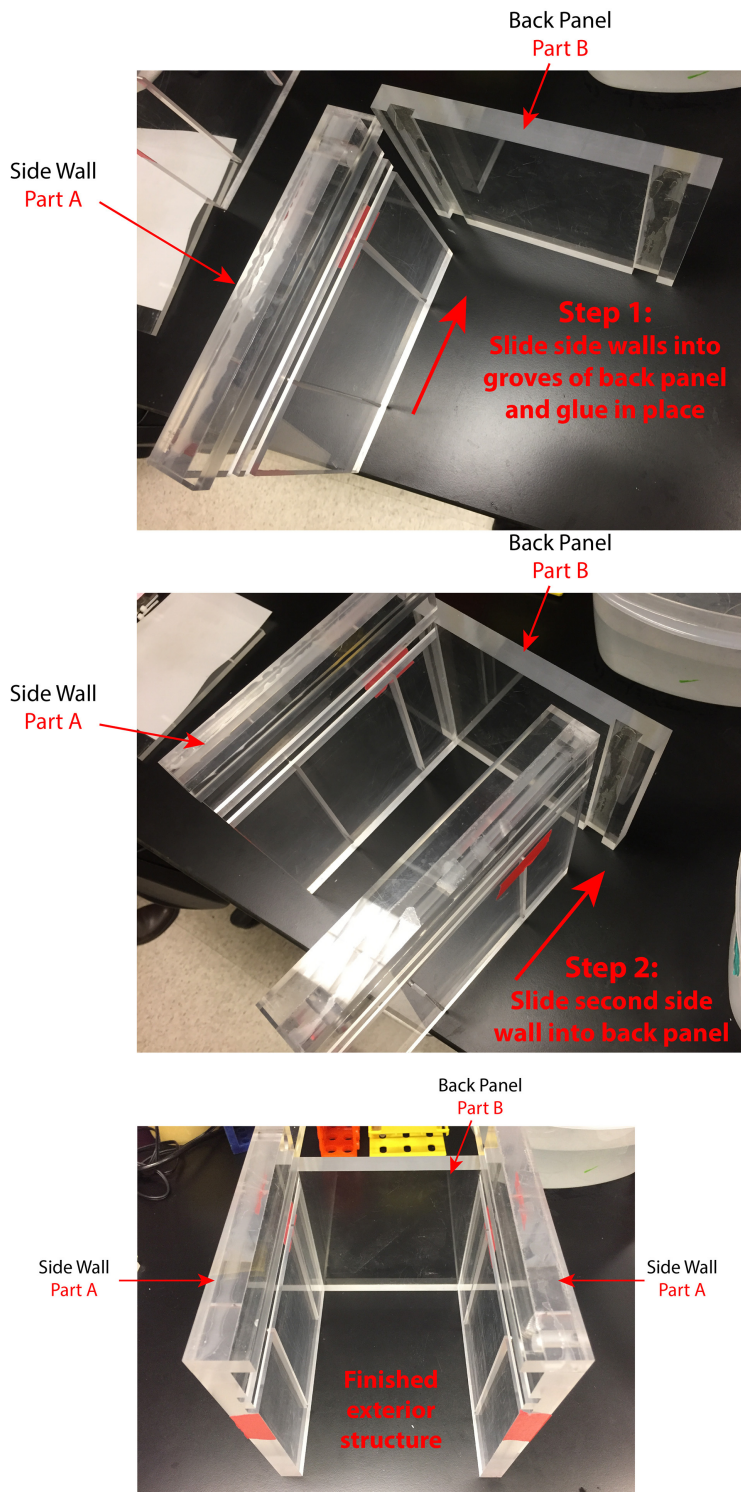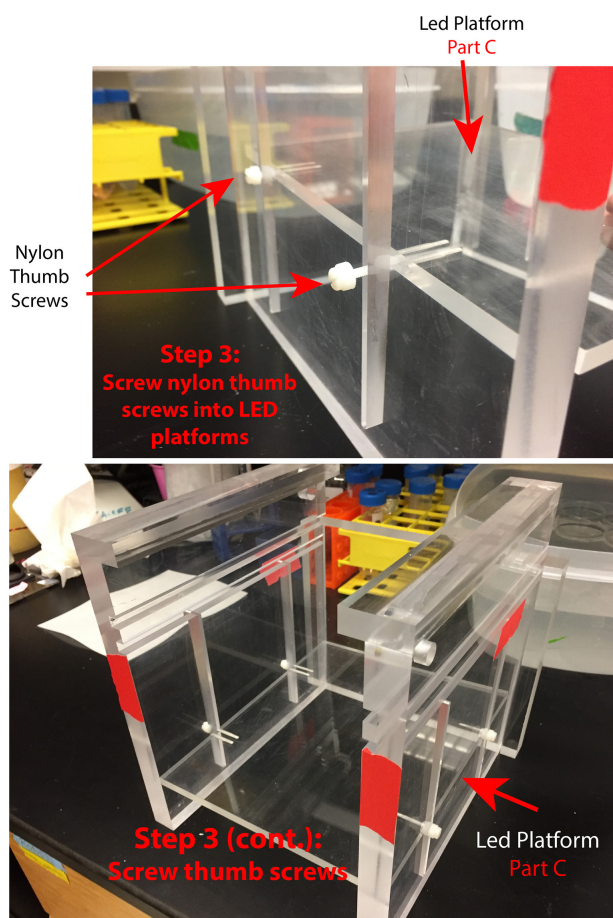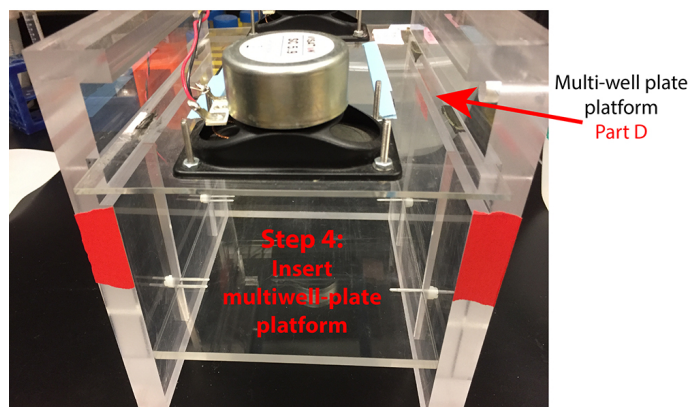i. Mount camera to P10 posts. XT66 rails should be constructed to hold IR mirrors (Figure 7).

Figure 1. zBox plans

Figure 2. Slide side walls into back panel grooves and epoxy in place

Figure 3. Use thumb screws to mount LED platforms into side wall slots

Figure 4. Insert multi-well plate platform into top grooves of side walls
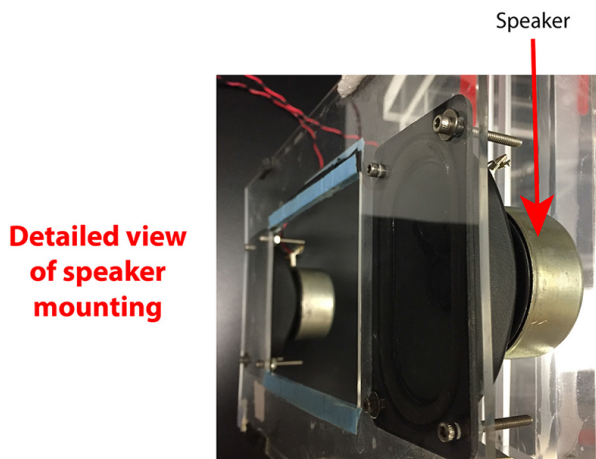
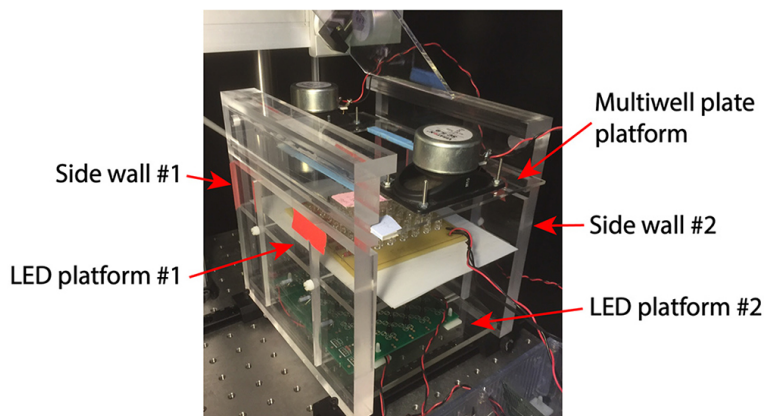**Figure 5. Detailed view of speaker mounting**
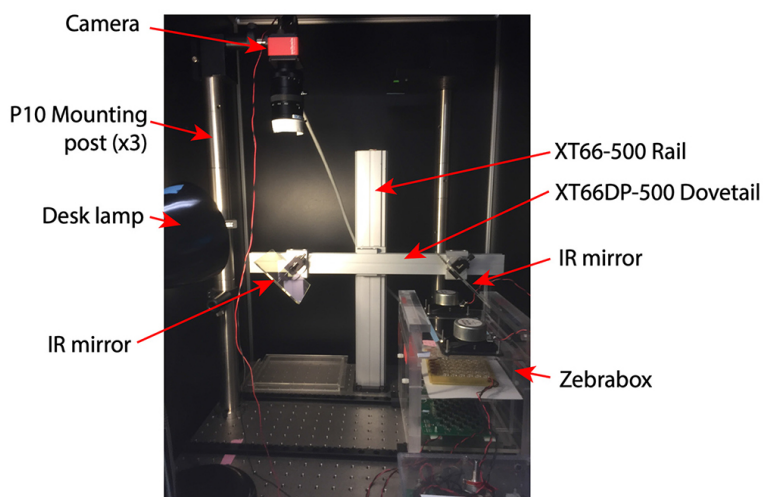

Figure 6. Completed zBox

Figure 7. Camera mounting

Software

1. Matlab2014a or later with Imaging Processing and Data Acquisition toolboxes installed

Procedure

1. Zebrafish embryos are raised in Petri dishes at low densities (~2 fish per ml) at 28.5 °C in E3 medium.

2. Larvae without inflated bladders and that do not respond to light taps on the dish on 4 dpf are removed.

3. Larvae are individually pipetted in 300 µl of E3 medium with a P1000 PIPETMAN pipettor, with the pipette tip cut to prevent damage to the larvae, and individually transferred from Petri dishes to the wells of a 48-well multi-well plate.

4. To prevent experimenter selection bias zebrafish larvae are randomly chosen for transfer to the 48-well plate.

5. 48-well microplate is transferred to a plexiglass box (Figures 4-7) kept inside an isolated dark chamber. Interior of chamber illuminated with constant diffuse white light is supplied by a 60 W bulb.

6. The behavioral apparatus is housed in a climate controlled environment at 22 °C.

7. If exposed to different treatments, larvae from different groups are tested simultaneously in the same micro-well plate.

8. Larvae used in single-day experiments are 6 dpf, whereas those used in the three-day long experiments are 5-7 dpf. For the three-day experiments, the larvae are kept in the microplates with 1,300 µl E3 overnight at 28.5 °C between measurements. The medium is refreshed the next day and then readjusted to 300 µl for the next habituation trial.

9. Behavioral activity within the microplate is recorded with a CCD camera, which is backlit by an array of infrared LEDs.

10. The behavioral assay consists of 15 min of acclimation to the high-throughput apparatus, followed by 10 min of spontaneous activity recording, and then the habituation protocol.

11. Sound stimuli are administered by two speakers mounted to the same platform as the microplate. The speakers are powered by a 15 W amplifier and delivered 900 Hz square waves of ~3 msec duration. Stimuli are delivered via a Native Instruments PCI-6229 DAQ controlled by MATLAB.

12. Habituation protocol consists of 10 stimuli at 90 sec ISI (0.4 V, ~95 dB) followed by 100 stimuli at 5 sec ISI (0.4 V, ~95 dB).

13. For each behavior experiment, data is analyzed from randomly sampled larval zebrafish derived from multiple clutches obtained on different days. We empirically determined that measuring behavior in approximately 100 larvae is sufficient to quantify behavioral variation at the population level.

Data analysis

1. Behavioral analysis is performed with MATLAB scripts (supplemental materials and Figure 8).
    README.txt file contains detailed information on how to run scripts. Basic skills in MATLAB
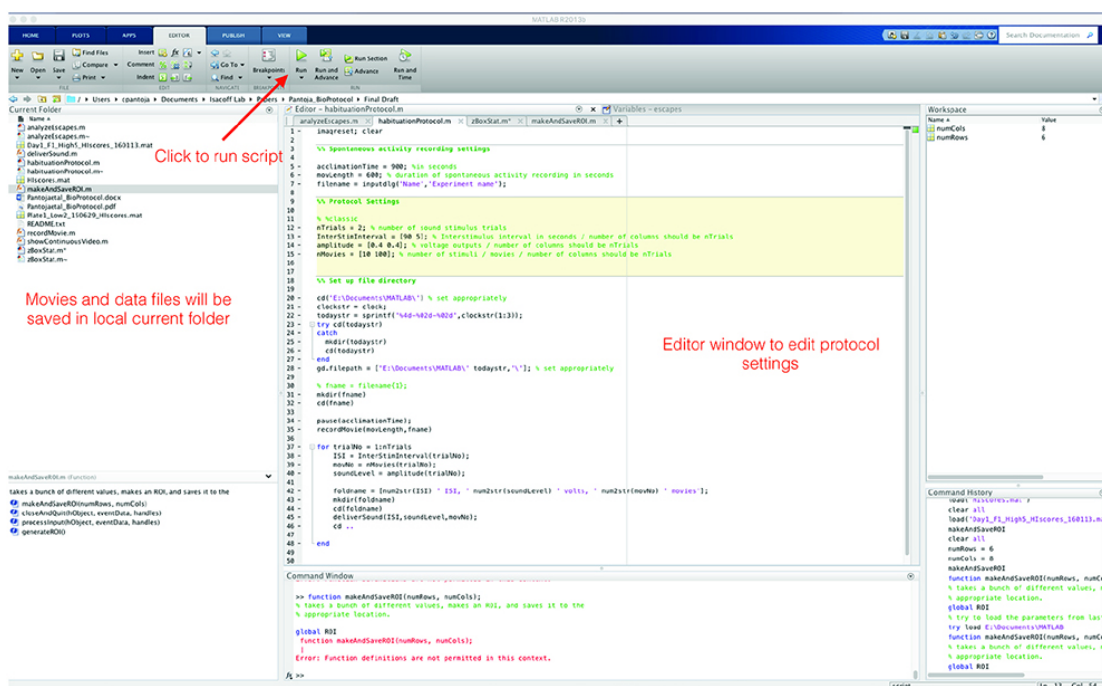   programming are recommended.

Figure 8. Screenshot of MATLAB user interface

2. Run habituationProtocol.m script. Escapes are detected using changes in pixel values between
    frames before and after sound stimulus presentation. An escape is counted if the difference of
    the integrated pixel values from the two frames immediately following the stimulus is statistically
    higher ($P < 0.01$, 2-sample t-test) than the distribution of pixel-change values taken from the
    non-escape portion spontaneous activity taken across all 120 movies (720 frames). The
    accuracy of this method was verified by visual inspection of the movies (Figure 9).
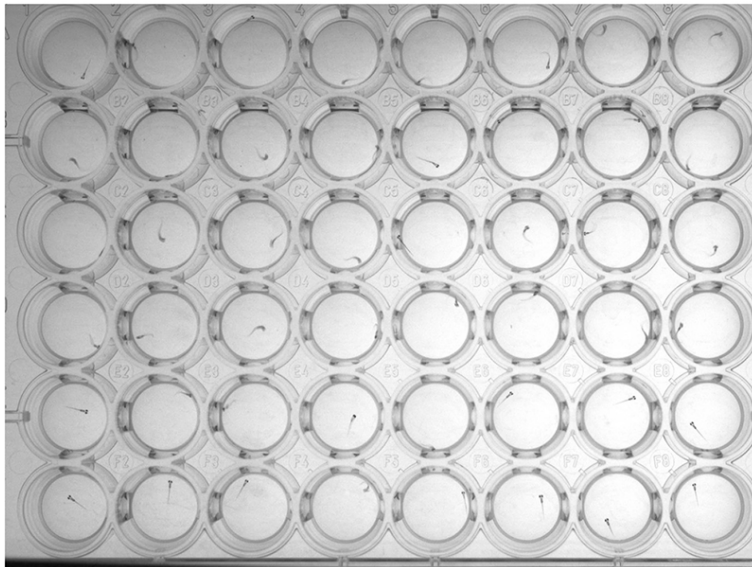
Figure 9. Representative image of 48-well plate during sound-induced escape

3. For analysis of behavioral movies run the analyzeEscapes.m script. We utilize the habituation index (HI) as a metric to describe acoustic startle habituation in individual larvae. The HI is defined as the difference in escape probability to the last 40 high-level stimuli under habituating conditions (10 sec inter-stimulus interval [ISI]) with the first 10 high-level stimuli under non-habituating conditions (90 sec ISI), divided by the probability of escape to the last 40 stimuli (HI =[P_{last40} - $P_{first10}$]/P_{last40}). Only highly-responsive fish, probability of response 90% or more to the initial 10 high-level stimuli, are used in calculating the HI (Figure 10).
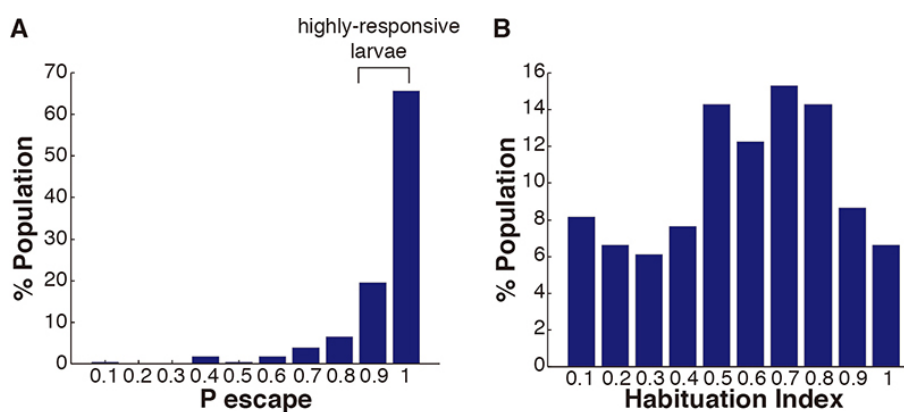
Figure 10. Graphic example of acoustic startle repose quantification. A. Frequency distribution of probability of escape values during the first 10 stimuli (ISI = 90 sec, n = 230) of habituation series. B. Frequency distribution of HIs for highly responsive fish in (A) (ISI = 10 sec, n = 196).

4. To determine if differences in HI between experimental groups are significant, we use the Mann-Whitney U-Test, which is included in the script zBoxStat.m. The HIscores.mat file in the supplemental materials provides an example of data to analyze.

Recipes

1. E3 embryo medium
   Note: It is not necessary to use filtered or sterile solutions.
   a. Making 50x stock solution:
      15 g instant ocean sea salt mix
      1 L DI H_2O
      Mix on stir plate for ~5 min
   b. Working solution:
      200 ml 50x E3
      10 L DI H_2O
      2 drops (~0.1 ml) of methylene blue

Acknowledgments

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