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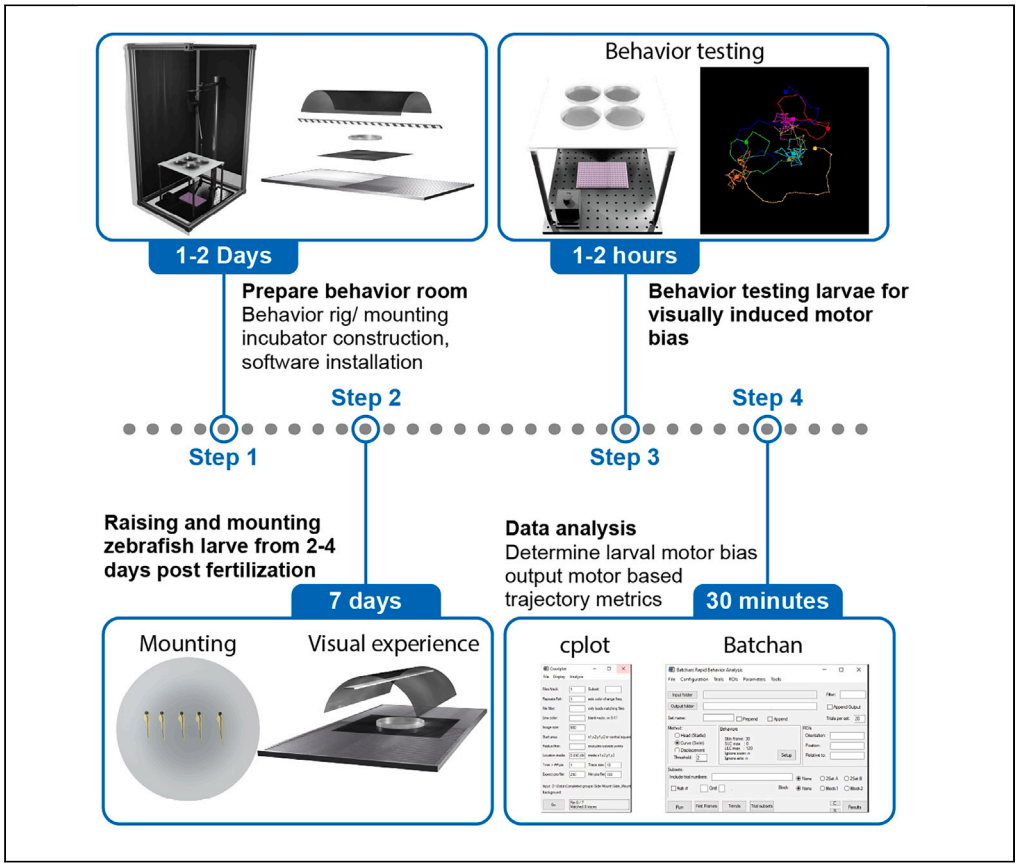
Protocol

Protocol for controlling visual experience during zebrafish development and modulation of motor behavior



Sensory experience instructs neurodevelopment and refines sensory processing. Here, we describe a minimally invasive protocol to immobilize zebrafish during early development to control visual experience. We describe how to prepare larvae for embedding in agarose at two separate timepoints in development. Then we describe how to build a behavior rig and use software to track zebrafish behaviors. Finally, we detail analyzing behavioral data to validate the protocol and determine outcomes of sensory dependent plasticity.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for controlling visual experience to one or both eyes

Visual control can be performed during different developmental windows

Flexible behavioral rig for recording photo-mediated larval zebrafish behavior

Straightforward programs for data analysis to describe zebrafish movement

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Protocol for controlling visual experience during zebrafish development and modulation of motor behavior

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SUMMARY

Sensory experience instructs neurodevelopment and refines sensory processing. Here, we describe a minimally invasive protocol to immobilize zebrafish during early development to control visual experience. We describe how to prepare larvae for embedding in agarose at two separate timepoints in development. Then we describe how to build a behavior rig and use software to track zebrafish behaviors. Finally, we detail analyzing behavioral data to validate the protocol and determine outcomes of sensory dependent plasticity.

For complete details on the use and execution of this protocol, please refer to Hageter et al. (2023).[1]

BEFORE YOU BEGIN

Protocol overview

This protocol will be a guide to immobilizing larvae during early development to provide controlled visual experience to a single eye (asymmetric experience) or both eyes (symmetric experience) and necessary equipment and protocols to detect changes in visuomotor responses. Here we describe methods where immobilized larvae will be exposed to a directional visual stimulus for a maximum of 48 h, which can be readily adapted to different timelines.[1] This protocol includes: 1. Raising and embedding larvae in low melting point (LMP) agarose along with removal and recovery from embedding, 2. Incubator configuration for providing visual stimulus, and 3. Visuomotor response testing, behavior rig assembly, and data analysis.

Institutional permissions

All experiments must be performed in accordance and with permission from the institutional animal care and use committee (IACUC). Protocols must include prolonged restraint of live animals as the experiments in this protocol required larval zebrafish to be mechanically restrained for 48 h.

Solutions to prepare

E3 media for raising larvae

Reagent	Final concentration (60×; mM)	Amount
Sodium chloride (NaCl)	300	17.52 g
Potassium chloride (KCl)	10.2	0.76 g
Calcium Chloride (CaCl)	19.8	2.91 g
Magnesium sulfate heptahydrate ($MgSO_4$-$7H_2O$)	19.8	4.88 g

(*Continued on next page*)

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Reagent	Final concentration (60×; mM)	Amount
Deionized H_2O	N/A	1 L
Total	**N/A**	**1 L**

1. Create 1 L of 60× E3 media stock.
2. Add 250 mL of 60× E3 stock solution to 15 L RO and mix on stir plate to create a 1× E3 solution.
3. Add 15 mL HEPES 7.5 pH buffer solution (Millipore: 7365-45-9) and pH to 7.4 with 1 M sodium hydroxide (NaOH)

Note: mixture before adjusting pH will be a pH of about 7.1–7.3. If you require large amounts of NaOH to adjust pH (>20 mL) you can adjust the pH of the 60× stock solution to 7.3–7.4 before mixing with RO.

Low melting point agarose solution

⚠ CRITICAL: Make this mixture on the day of mounting (See steps 1–11). If this solution is made too far in advance, the agar will cool unevenly and increase chances of damaging larvae when manually orienting with a pipette.

4. Add 1 g NuSieve™ GTG™ Agarose (Lonza: 50081) to 100 mL 1× E3 to create a 1% agar solution.
5. Dissolve agar by microwaving for 60 s.
6. Let cool at room temperature (20°C–22°C) until the solution is 50°C–55°C.
7. Store in an incubator at 55°C until ready for use.

Set up incubator for controlled visual experience

Timing: 45 min

This portion of the protocol describes construction of the incubator used to provide asymmetric visual experience for the duration of the critical period (2–4 dpf). By completing this section, you will have a reliable way to control visual experience to larval zebrafish (Figure 1).

8. Separate two shelves in a light controlled incubator by at least 6″.

Note: The incubator should have an exterior which prevents external light from entering and is suitable for housing zebrafish larvae.

9. On the bottom shelf, place a dark surface.

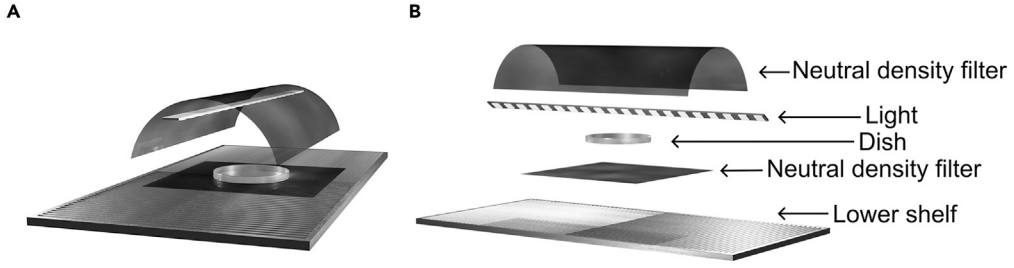

Figure 1. Mounting incubator assembly
(A) Representative diagram of complete mounting set up in incubator.
(B) Deconstructed parts for mounting incubator. The top shelf is excluded.

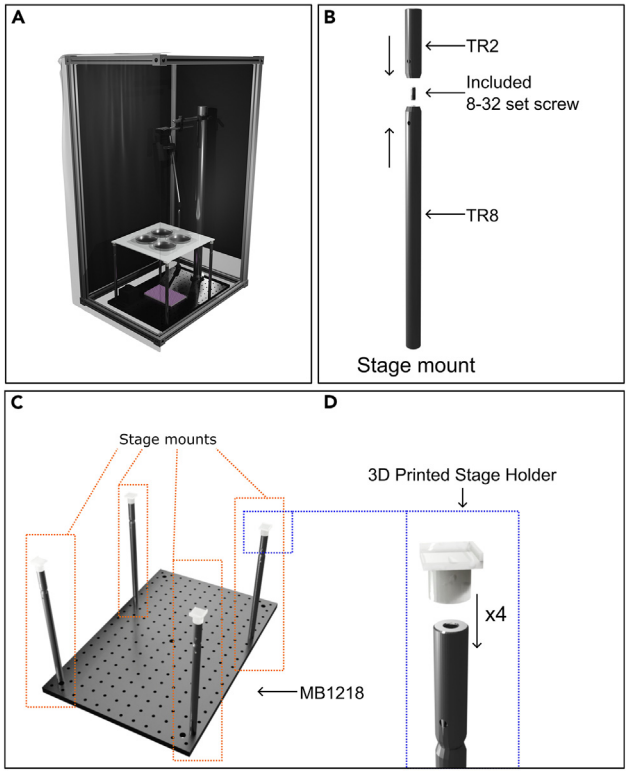

Figure 2. Behavior rig assembly (Stage mounts)

(A) Complete behavior rig and blackout box. Forward views made transparent for visualization of the interior.

(B) Individual stage mount construction using a 2 in. optical post (TR2) and an 8 in. optical post (TR8) along with the included 8–32 set screw.

(C and D) (C) Breadboard (Thorlabs: MB1218) and stage set-up for behavior rig. Shows breadboard with stage mount assembly. (D) Inlay (blue) shows location of 3D printed stage holder placement for recording platform (HDPE material) stabilization.

Note: This can either be a non-reflective piece of black foam core hardboard or a neutral density filter. This is used to minimize light reflections from the overhead light beneath the petri dish.

10. Attach a non-diffused LED strip light controlled by a dimmer switch and timer to the underside of the upper shelf.

Note: This will be used to control light intensity and day/night cycles.

11. Set the intensity of the light so that at the level of the bottom shelf it is ~80 μW/cm^2.
    a. This can be adjusted using the dimmer switch and measured using an optometer (International Light Technologies: ILT2400).
12. Set the incubator to 28.5°C and the timer to a 14 h on, 10 h off day night cycle.
13. Insert a neutral density (ND) filter between the light strip and upper shelf so the ND filter drapes over either side of the light. This will reduce reflections from the interior walls of the incubator.

Behavior rig assembly

Timing: 1 day

This section describes how to build a behavior rig used for tracking larval zebrafish. The behavior rig will be modular as to be applied to other paradigms if needed.

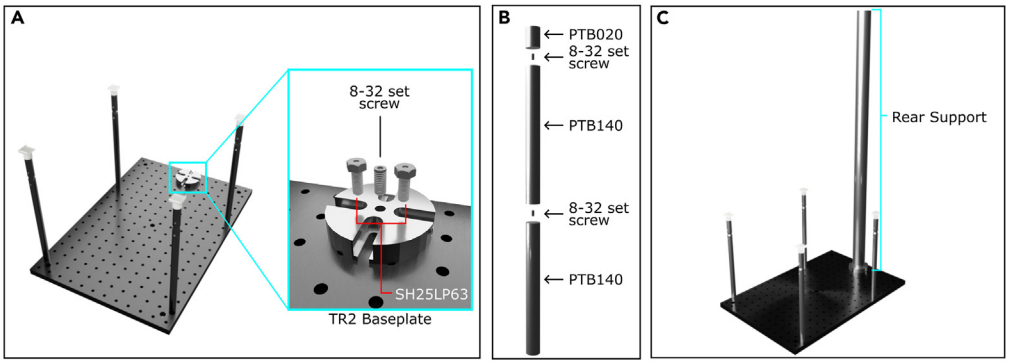

Figure 3. Rear support construction

(A) The rear support baseplate (TR2) assembly is fastened to the breadboard using $^{1}/_{4}$″-20 low profile channel screws (SH25LP63 5/8″ length). Inlay (Cyan) depicts the location where screws should be inserted. An 8–32 set screw is inserted into the middle threads of the TR2 baseplate to fasten the rear support.

(B) The rear support is constructed using two 1.5″ diameter, 14″ length, stainless steel posts (PTB140) and one 1.5″ diameter, 2″ length, stainless steel post (PTB020) using 8–32 set screws.

(C) Representative of completed rear support post construction.

Note: a list of materials required to build one behavior rig is included (key resources table; *** indicates items that cannot be substituted in the protocol; (n) following name indicates amount of item needed). The completed behavior rig occupies a space of 16″x22″x36″ (Figure 2A). Ensure there is enough space that the behavior rig sits completely on a flat, solid surface and there is enough excess space for a desktop and monitor, or laptop.

14. Place breadboard (ThorLabs: MB1218; 12″x18″ dimensions) on top of $^{1}/_{4}$″-thick vibration dampening pad (McMaster-Carr: 5940K58).

Note: For reference the breadboard should be oriented with the 12″ side facing the front and rear. This will be used for reference in the following steps. The breadboard and vibration dampening pad house all structural components in the following steps while isolating the behavior rig from external stimuli which could alter behavior.

15. Attach an 8″ optical post (ThorLabs: TR8) to 2″ optical post (ThorLabs: TR2) using included 8–32 screws (x4) (Figure 2B).
16. Using nylon plastic flat tip set screws (McMaster-Carr: 94564A090) attach 4 optical posts to breadboard in 12″x12″ square by screwing them into the breadboard.

Note: This will be referred to as the stage mount (Figure 2C).

17. Use provided "StageHolder.stl" (See supplemental) file to 3D print four stage holders to attach to the top of the stage mounts (Figure 2D). The stage holder should fit loosely to the blunt end of the optical post.

Note: These will be used to hold the stage in place to prevent petri dish movement when completing multiple rounds of behavior.

18. Using two alloy steel low-profile socket head screws (ThorLabs: SH25LP63) attach mounting post base (ThorLabs: PB2) in central rear of breadboard at least 2″ back from optical posts (Figure 3A).
19. With stainless steel cup point set screws (McMaster-Carr: 92311A540) attach two 1.5″ diameter stainless steel posts, length 14.0" (ThorLabs: PTB140) together with another 1.5″ diameter stainless steel post, length 2.0" (ThorLabs: PTB020) on one end (Figure 3B).

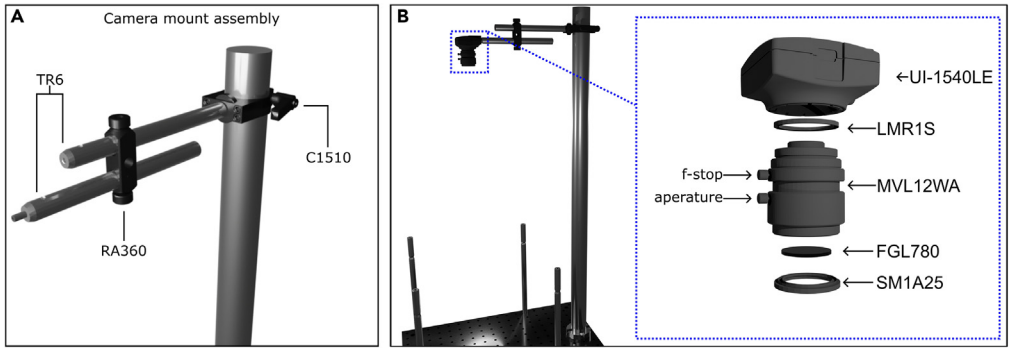

Figure 4. Camera mount assembly

(A) Camera mount assembly completed using two 6″ optical posts (TR6) inserted into a parallel clamp (RA360) and attached to the rear support with a 1.5″ diameter post mounting clamp with quick release handle (C1510).

(B) Location where camera should be installed on the exposed end of the TR6 optical post for the camera mount. Inlay (blue) depict lens assembly. To the camera (UI-1540LE) attach a 1″ lens mount to hold the 12 mm C-mount camera lens. The 780 nm longpass filter (FGL780) is attached to the lens using an SM1 thread adapter (SM1A25).

Note: This entire post will be mounted to the PB2 mounting base with the 2″ post on top. From here on this will be referred to as the rear support post (Figure 3C).

20. Attach two 6″ optical posts (ThorLabs: TR6) to a parallel clamp (ThorLabs: RA360) with tapered ends facing the same direction. To the tapered end on one post attach an IDS UI-1540LE mono USB 2.0 camera (IDS: EV76C560ABT). To the blunt end of the opposite post attach the compact post mounting clamp with quick release handle (ThorLabs: C1510).

Note: This assembly will be referred to as the camera mount assembly.

21. Attach the camera mount assembly to the rear support post using the quick release handle to tighten (Figure 4A).
    a. Adjust posts within the parallel mount on the camera mount assembly so that the camera is centered among stage mounts.
22. Build the lens and attach it to the camera (Figure 4B). Place a 780 nm long-pass filter (ThorLabs: FGL780) on the internal thread lock of an adapter (ThorLabs: SM1A25) and screw into lens, then attach the assembly to the camera using a C-mount thread adapter (ThorLabs: MVL12WA).
23. Attach an optical post holder with spring loaded hex-locking thumbscrew (ThorLabs: PH1) to the left of the rear support 2″ from the left edge of the breadboard with a steel cup point set screw (3/4″) (Figure 5B).
    a. Place a 12″ optical post (Thorlabs: TR12) within the post holder so the tapered end is exposed.
    b. To the tapered end attach a locking ball and socket mount (ThorLabs: TRB1) followed by attaching an 8″ optical post (Thorlabs: TR8) to the other end of the socket mount (Figure 5C).

Note: This will be referred to as the LED support.

24. 1″ from the top optical post on the LED support, attach a rotating clamp (ThorLabs: SWC/M) and orient perpendicular to the rear support (Figure 5D).
    a. Within the open socket add a 4″ optical post with the tapered end facing away from the LED support.
    b. Cut out a circle of 1/32″ high density polyethylene (HDPE; McMaster-Carr: 8619K421) in a 1″ diameter and place into tube lens using internal threads to hold in place.

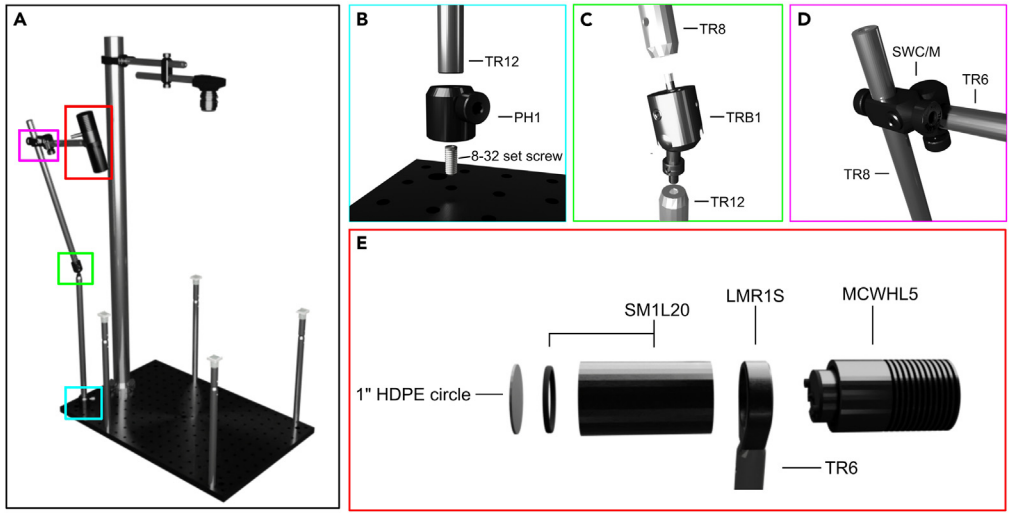

Figure 5. Light mount assembly

(A) Representative light mount assembly installation. Colored boxed correspond with panels (B–E).

(B) (Cyan) The 1.5″ optical post holder (PH1) is attached to the breadboard using an 8–32 set screw. This will hold the 12″ optical post (TR12).

(C) (Green) The rotating clamp (TRB1) is attached to the tapered end of the 12″ optical post (TR12) depicted in (B) An An 8″ optical post (TR8) is attached to the other end of the rotating clamp.

(D) (Magenta) The 8″ optical post depicted in (C) holds the rotating clamp (SWC/M) with a 6″ optical post attached to the open end. The complete assembly is oriented perpendicular to the 8″ optical post.

(E) (Red) The LED assembly includes the LED (MCWHL5) connected to a 1″ diameter lens mount (LMR1S). To the lens mount, attach a 2″ tube lens with included retaining ring (SM1L20) and the 1″ HDPE cutout to the end to create diffuse illumination.

Note: This set-up provides diffuse light to equally cover the stage whereas the light directly from the LED tends to produce a "spotlight" effect without the HDPE in place.

25. Attach both the 6500K LED light (ThorLabs: MCWHL7) and 2″ SM1 lens tube (ThorLabs: SM1L20) to a 1″ Lens mount (ThorLabs: LMR1S) and screw into the tapered end of the 4″ optical post attached to the LED support (Figure 5E).
26. Into the T-Cube LED driver, plug in the LED and power source (Thorlabs: LEDD1B; Thorlabs: KPS201). Adjust the dial so the arrow on the front of the T-Cube points to 1.0 mA (Figure 6A).
    a. Wire together a BNC connector (Gravitech: BNC-F-TERM-S) with positive and negative leads and plug it into the T-Cube.
    b. The connecting ends should be wired into the DAQ-Module (National Instruments: USB-6001) with positive wire going into the P0.1 pinout and negative into D GND (Figure 6B).
27. Plug in the infrared light (IR) and place centered among the stage mounts (Figure 7A).
28. Place T-Cube and Daq Module adjacent to the behavior rig, but not within the stage mount area.
29. Place 12″x12″x1/8″ HDPE on the stage mounts (Figure 7B).

Note: This is where the petri dishes for behavior testing will be placed. The HDPE should lay flat on the stage holders and not move around if placed correctly. Using an HDPE stage allows for diffusion of IR light from beneath to create an equally illuminated area for tracking larvae.

30. Plug the DAQ-Module and camera into the USB-A ports on a computer.
31. To construct the dark box encasing the behavior rig create two rectangles by attaching the corner plastic cubes (BaseLab Tools: X2020-C3W-P) to 21″ and 15″ construction rails using low profile socket screws (Thorlabs: SH25LP63). Attach the rectangles together with the 36″ construction rails.

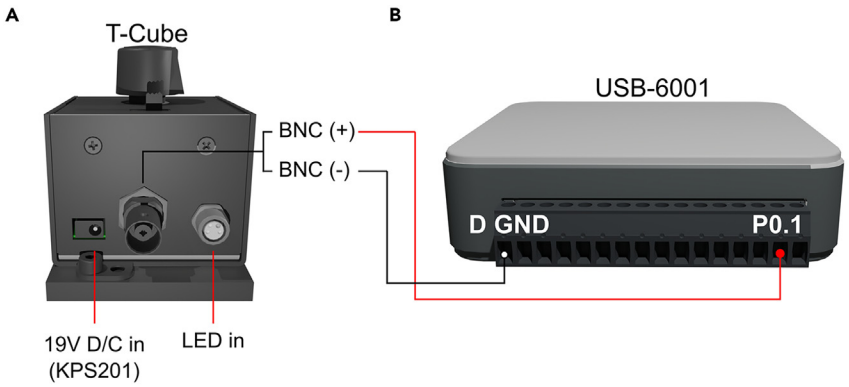

Figure 6. T-Cube and DaqModule setup

(A) Depiction of T-Cube LED controller (Thorlabs: LEDD1B). Plug in power source (Thorlabs: KPS201) to the indicated input. The LED gets plugged into "LED in". To the center plug attach the BNC connection with a positive (+) red wire and negative (-) black wire.

(B) Representative of DaqModule (National Instruments: USB-6001) pinouts. The positive wire plugs into the pin 1 port (P0.1) and the negative to the digital ground port (D GND). Refer to the pinout diagram included with the DaqModule to ensure correct placement.

32. Cut out foam core hardboard to fill the top 21″x15″ rectangle and 3 of the 36″x15″ sides.

    Note: The construction rails have a $^{1}/_{4}$ in. slot along each side. Blackout board should be cut $^{1}/_{4}$ in. longer on each side to account for this. The blackout board should fit snugly into the construction rails and if multiple pieces are used, they should be flush.

33. Cut a ~5″ section out of the rear foam core hardboard to allow area for wires to exit the dark box.
34. Fill any gaps by using 2″ blackout tape.
35. Using drop-in T-nuts (BaseLab Tools: X2020-DT-M5-P10), attach blackout cloth (Thorlabs: BK5) to the top construction rails and drape over the front opening of the blackout box.

    Note: Over time with use the blackout cloth tends to stretch. By using the drop in T-nuts, the cloth length can be adjusted to account for this wear.

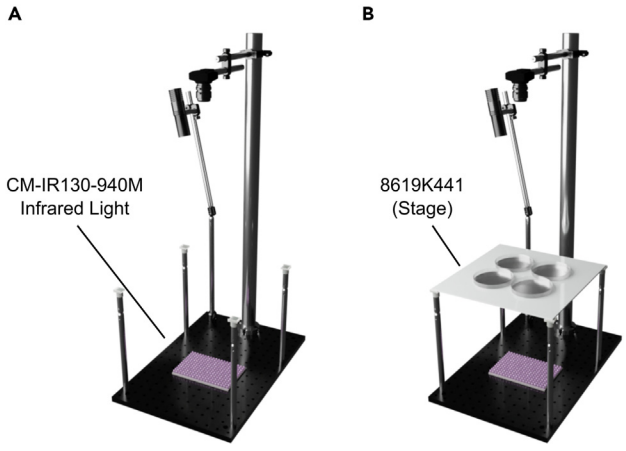

Figure 7. IR and stage placement

(A) Representative depiction of the infrared light placement (CMVision: CM-IR130-940M) centered among the four stage mounts.

(B) Representative image of HDPE (McMaster Carr: 8619K441) stage placement set in 3D printed stage holders attached to the stage mounts.

36. Attach the blackout cloth to the frame using Velcro strips or tape.

Software setup

Timing: 20 min

This section describes the setup and use of the software required to complete this protocol. Major programs include IDL, uEye, and NiMAX, which will allow for control of stimulus series and tracking of larval zebrafish.

37. Install IDL (Interactive Data Language, NV5 Geospatial, Inc.). This is the integrated development environment that allows you to run IDL programs.
38. Install drivers for uEye camera (found at https://www.ids-imaging.us).
    a. Make sure the camera works by testing in uEye application. In the application select "Live" which will open a window and display the current view of the camera. The image will be very dark due to the IR filter attached to the lens. Aim the camera at a normal or IR light and you will be able to see the image clearer.
39. Install NiMax (found at https://www.ni.com/). This program contains the necessary drivers for the Daq module along with a user interface which allows testing for hardware faults and functionality.
    a. Test to make sure Daq Module is functioning by using the test panels in NiMax. By switching the number 1 pinout to "High" this will send a signal to the T-Cube to turn on the light. Make sure T-Cube switch is set to "Trig" and light is turned on by adjusting the dial on top of the T-Cube.
40. Download "ftrack" folder and place it on C:/ drive. This is a storage folder which contains default configurations for scripts ran in EventTimer.sav. All program files are listed in the supplemental data repository (https://doi.org/10.17632/gmknc9wvwt.1).

⚠ CRITICAL: ftrack folder must remain on C:/ drive to be recognized by the programs used in this protocol.

41. Download "DaqModule" folder and place it on C:/ drive. This folder contains applications used for tracking and data generation (EventTimer.sav, Et.sav, batchan.sav, and cplot.sav)

⚠ CRITICAL: These cannot be removed from the C:/ drive or IDL applications will not function properly. These programs have been tested on Windows 7/10 but are unknown for Linux or macOS builds.

42. Create shortcuts for each application described in step 30 and move to a desired location.
43. Open "Et".
44. Using the provided IDL scripts test to ensure the Light and camera were installed properly by using "LightTest" and "173_explore_setparams_multiplex". You will visualize the following:
    a. LightTest: The console window will open, the LED will turn on for 1 s, turn off for 1 s, and turn back on. When the script is finished running, the console window will close.
    b. 173_explore_setparams_multiplex: You will see a greyscale image produced by the camera. Wave an object in view of the camera to ensure the camera is capturing video. If the image stutters and you receive an error in the console window ("Snapshot error 178"), quit the script by closing the console window. On the top bar of the Et window click "Configuration" and make sure "Slow Computer" is turned on. Rerun the script and the error should disappear.

Note: the full development environment for IDL is a paid-for product, but the software can be downloaded and used without product license verification. For the purposes of this protocol, purchase of IDL is not required.

Figure 8. Setting equal contrast behavior arena

(A) Camera image obtained from running "173_setparams_multiplex" script representing desired contrast. Numbered circles indicate regions clicked denoted in the console window. Regions clicked indicate x and y coordinates along with the gray value for the pixel clicked.

45. Next you will need to adjust the camera's view to create an equal contrast of light in the viewing field. Adjust the lens' f-stop dial (See Figure 4B) until the array of bulbs on the IR is visible, then cut out 8 × 5″ rectangles of HDPE to place on top of the IR until an equally contrasted image is seen in the camera view (Figure 8A).

Note: by clicking anywhere on the camera view window the console log will display the xy-coordinates of the pixel clicked along with the gray value (0–255). Click on a few regions around the view window and ensure the gray value does not deviate more than 175 units from any other point.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NuSieve™ GTG™ Agarose***	Lonza	50081
Ethyl 3-aminobenzoate methanesulfonate (tricane)	Millipore Sigma	E10521
Calcium Chloride Dihydrate	Fisher Scientific	10035-04-8
Magnesium sulfate heptahydrate	Millipore Sigma	10034-99-8
HEPES	Millipore Sigma	7365-45-9
Sodium Chloride	Promega	7647-14-5
Potassium chloride	Millipore Sigma	7447-40-7
Deposited data		
Original publication data	Mendeley	https://doi.org/10.17632/bpj99zrshg.2
New data and software	Mendeley	https://doi.org/10.17632/gmknc9wvwt.2
Experimental models: Organisms/strains		
TL Wildtype zebrafish	ZIRC	ZL86
Software and algorithms		
IDS uEye***	https://www.ids-imaging.us/download-details/AB02536.html	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NiMAX***	https://www.ni.com/en-us/support/downloads/drivers/download.system-configuration.html#479580	N/A
IDL***	https://www.l3harrisgeospatial.com/Software-Technology/IDL?gad=1&gclid=CjwKCAjwsvujBhAXEiwA_UXnABNVGgqtRcDgIm2M5H9KuYQDLwJ3j4DPpyxAM2RuIa72FwCtbDvrexoCRSMQAvD_BwE	N/A
Cplot***	Mendeley data: https://doi.org/10.17632/gmknc9wvwt.1	
Et***	Mendeley data: https://doi.org/10.17632/gmknc9wvwt.1	
Batchan***	Mendeley data: https://doi.org/10.17632/gmknc9wvwt.1	
Other		
X2020 Construction Rail - 36 In. (4)	Base Lab Tools	X2020-36
X2020 Construction Rail - 15 In. (4)	Base Lab Tools	X2020-15
X2020 Construction Rail - 21 In. (4)	Base Lab Tools	X2020-21
X2020 Drop-In T-Nuts - M5 Thread (10 Pk)	Base Lab Tools	X2020-DT-M5-P10
X2020 Black Plastic Corner Cubes For Enclosures (Set Of 4) (2)	Base Lab Tools	X2020-C3W-P
Foam Core Hardboard 20in × 30in × 3/16in (6)	Base Lab Tools	X2020-HB20X30
Ø1.5 In. Stainless Steel Post - 14 In. (2)	Base Lab Tools	PTB140
Ø1.5 In. Stainless Steel Post - 2 In.	Base Lab Tools	PTB020
Mounting Post Base, Ø2.40″ × 0.50″ Thick	Thorlabs	PB2
Compact Ø1.5″ Post Mounting Clamp, Included Quick-Release Handle, Imperial	Thorlabs	C1510
Aluminum Breadboard 12″ × 18″ × 1/2″, 1/4″-20 Taps	Thorlabs	MB1218
Ø1/2″ Optical Post, SS, 8–32 Setscrew, 1/4″-20 Tap, L = 12" (2)	Thorlabs	TR12
Ø1/2″ Optical Post, SS, 8–32 Setscrew, 1/4″-20 Tap, L = 6" (2)	Thorlabs	TR6
Ø1/2″ Optical Post, SS, 8–32 Setscrew, 1/4″-20 Tap, L = 8" (4)	Thorlabs	TR8
Ø1/2″ Optical Post, SS, 8–32 Setscrew, 1/4″-20 Tap, L = 4″	Thorlabs	TR4
Ø1/2″ Optical Post, SS, 8–32 Setscrew, 1/4″-20 Tap, L = 2" (4)	Thorlabs	TR2
Ø1/2″ Post Holder, Spring-Loaded Hex-Locking Thumbscrew, L = 1″	Thorlabs	PH1
Parallel Clamp for Ø1/2″ Posts, #8 Counterbore and 3/16″ Hex	Thorlabs	RA360
Rotating Clamp for Ø1/2″ Posts, 360° Continuously Adjustable, 5 mm Hex	Thorlabs	SWC/M
12 mm EFL, f/2.8, for 1/2″ C-Mount Format Cameras, with Lock***	Thorlabs	MVL12WA
Ø25 mm RG780 Colored Glass Filter, 780 nm Longpass***	Thorlabs	FGL780
Adapter with External M25.5 × 0.5 Threads and Internal SM1 Threads	Thorlabs	SM1A25
6500 K, 930 mW (Min) Mounted LED, 1300 mA***	Thorlabs	MCWHL7
T-Cube™ LED Driver with Trigger Mode, 1200 mA Max (Power Supply Not Included)***	Thorlabs	LEDD1B
15 V, 2.66 A Power Supply Unit with 3.5 mm Jack Connector for One K- or T-Cube	Thorlabs	KPS201
Ø1″ Lens Mount with Internal and External SM1 Threads, 8–32 Tap	Thorlabs	LMR1S
1/4″-20 Low-Profile Channel Screw, 5/8″ Long, 50 Pack	Thorlabs	SH25LP63
Black Masking Tape, 2″ × 180′ (50 mm × 55 m) Roll	Thorlabs	T137–2.0

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SM1L20 - SM1 Lens Tube, 2.00″ Thread Depth, One Retaining Ring Included	Thorlabs	SM1L20
Locking Ball and Socket Mount, 8–32 Threaded Base and Setscrew	Thorlabs	TRB1
Black Nylon, Polyurethane-coated fabric, 5′ × 9′ × 0.005″	Thorlabs	BK5
Vibration-Damping Pad for Heavy Machinery, 12″ Long × 12″ Wide × 1/4″ Thick	McMaster-Carr	5940K58
18-8 Stainless Steel Cup-Point Set Screw, 1/4″-20 Thread, 3/4″ Long	McMaster-Carr	92311A540
18-8 Stainless Steel Cup-Point Set Screw, 1/4″-20 Thread, 1/2″ Long	McMaster-Carr	92311A537
Nylon Plastic Flat-Tip Set Screws, 1/4″-20 Thread, 3/4″ Long	McMaster-Carr	94564A090
Moisture-Resistant HDPE Sheet, 12″ × 12″ × 1/16" (3)***	McMaster-Carr	8619K421
Moisture-Resistant HDPE Sheet, 12″ × 12″ × 1/8" (3)***	McMaster-Carr	8619K441
8 AI (14-Bit, 20 kS/s), 2 AO (5 kS/s/ch), 13 DIO USB Multifunction I/O Device***	National Instruments	USB-6001
USB Cable	National Instruments	782909–01
UI-1240LE***	IDS	EV76C560ABT
CMVISION INVISIBLE FOR HUMAN EYE 198PC LEDS 50–100 FT LONG RANGE IR ILLUMINATOR (3A 12VDC POWER INCLUDED)***	CMVision	CM-IR130-940NM
BNC-F-TERM-S	Gravitech	BNC-F-TERM-S
W-Series (Wide) Flexible LED Strip Light - Standard Bright (9 LEDs/foot)	aspectLED	SKU AL-SL-W-S
Reflective Neutral Density (ND) Filters (4)	Edmund Optics	#64-137
Single Color Mini Rotary Dimmer	LEDSupply	GL-DIM1002
1 ft DC Power Cable 5.5 × 2.1 mm Female Pigtail Female Plug	Cable Leader	SE104-7601
Wall Adapter Power Supply - 12 VDC, 600 mA (Barrel Jack)	SparkFun	TOL-15313
TiFFCOFiO Indoor Mechanical Outlet Timer, 3 Prong Timers for Electrical Outlets, 24-h Programmable Plug in Light Timer, ETL Listed, 2 Pack	Amazon	N/A
20 Gauge 2 Pin Extension Wire, EvZ 20 AWG 2 Conductor Parallel Electric Cable Cord for Led Strips Single Color 3528 5050, Red Black, 33 ft/10 M	Amazon	SYNCHKG060430
Hand-held light meter/optometer	International Light Technologies	ILT2400
Dumont #5 Forceps (2)	Fine Science Tools	11251–10
Double Spatula, 8.07 in overall length, 3.94 in handle length, silver	Grainer	21RL37
Falcon 40-μm Cell Strainer, Blue, Sterile, Individually Packaged 50/Case	Corning	352340

STEP-BY-STEP METHOD DETAILS

Mounting zebrafish in low melting temperature agarose from 2-4 dpf

Timing: 2 days for collecting and raising larvae, 1–2 h for mounting (dependent on the number of individuals mounted)

This section describes the method for embedding zebrafish larvae in low melting point agarose to be exposed to asymmetric visual experience for ~42 h. This method is used to induce a sensory dependent motor bias on larval zebrafish for behavior testing at 7 dpf.

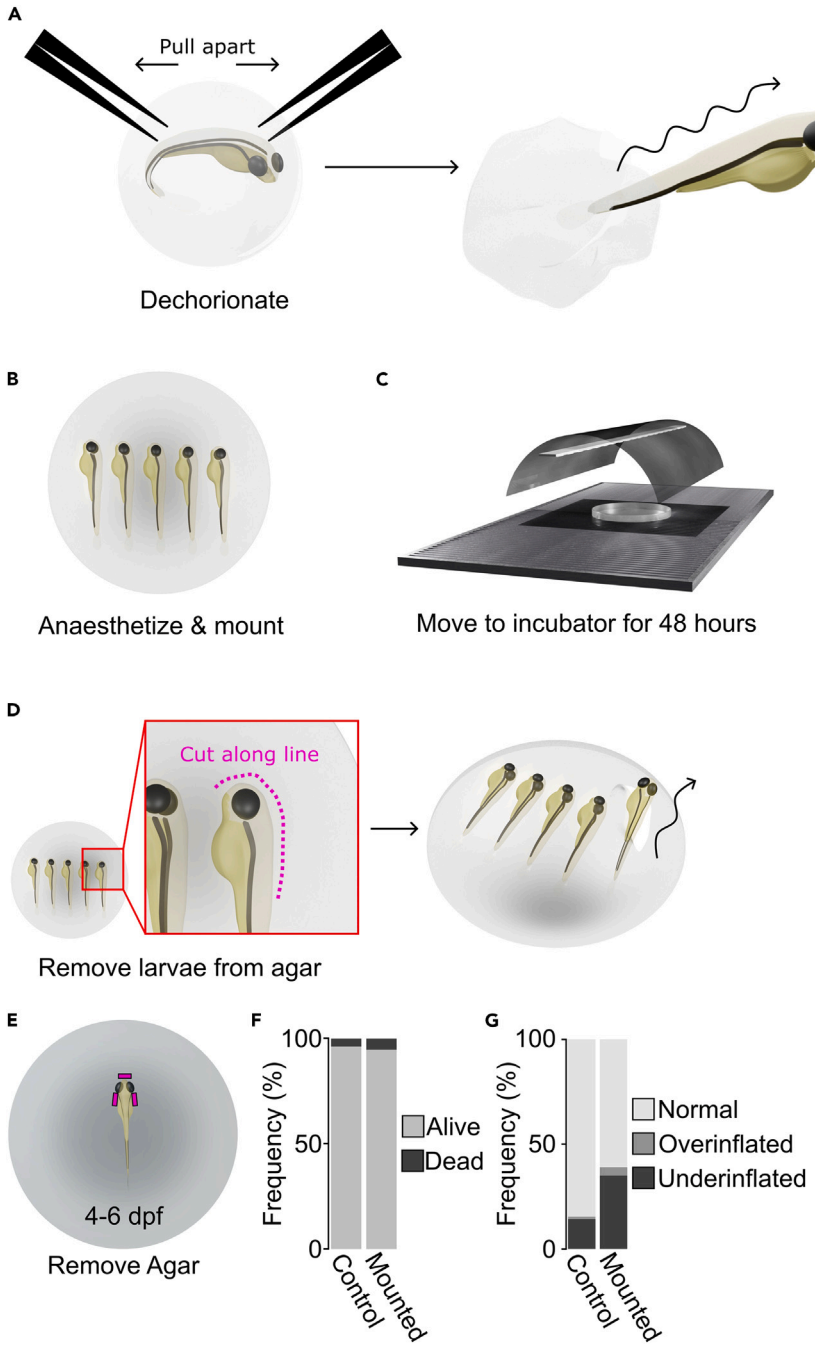

Figure 9. Embedding larvae in LMP agarose

(A) Representative image of dechorionating process by using microdissection forceps to pull apart chorion so larvae can swim away freely.

(B) Schematic of multiple larvae mounted in a single direction (Right side down) after anaesthetizing in 1% tricane (Millipore: E10521).

(C) Embedded larve are moved to the prepared mounting incubator. (Refer to "Before you begin" steps 8–13).

(D) Representative of removing larvae from agar using microdissection forceps. Inlay (red) shows recommended agar cut location (Magenta dotted line) to free larvae from the agar.

(E) Location of agar removal (magenta) for embedding from 4-6 dpf to allow for adequate oxygen and ion exchange during mounting.

Figure 9. *Continued*

(F) Lethality of mounting for unmounted control (N = 175) and mounted (N = 198) larvae. Controls were anesthetized for the same duration as mounted individuals, but not embedded in LMP agarose following. Lethality counts were collected at 7 dpf.

(G) Same experiment as in (F) examining swim bladder inflation rates. Overinflated represents individuals who float at the surface of the water, underinflated represents individuals who cannot maintain buoyancy, and normal inflation consists of individuals who can freely swim throughout the water column.

1. Collect embryos and house at a density of 40-60 individuals per 10 cm Petri dish or 10–20 per 6 cm dish. Embryos should be collected immediately after fertilization and kept in E3 embryo media (see steps 1–3).
2. Embryo media (E3) should be exchanged daily, and all developmentally delayed, dead, or deformed individuals removed.
3. At 48 h post fertilization (hpf) manually dechorionate individuals with dissecting forceps (Fine Science Tools: 11251-10) under a dissecting microscope (Figure 9A). All larvae that are damaged in dechorionating should be removed.
4. Anaesthetize individuals in 0.1% tricane (Millipore: E10521) dissolved in E3 media for 1–2 min.

⚠ CRITICAL: Only anaesthetize the individuals which are about to be mounted.

5. Transfer 5–10 individuals to a new dish in a small drop of embryo media using a Pasteur pipette.
6. Immediately apply a thin layer (0.5–1.0 mL) of 1% LMP agarose dissolved in E3 media over the larvae using a 3 mL Pasteur pipette.
   a. Agar drop should take up about 25% of the dish if immobilizing in a 6 cm dish. Larvae will be submerged in the agar which will provide oxygenation for a short time in the absence of E3 media.

⚠ CRITICAL: Larvae should be returned to E3 media no later than 20 min after being mounted. The agar will begin to dry out and increase lethality.

7. Using microdissection forceps or a p10 micropipette tip, arrange individuals to either have the left, right, or both eyes facing up (towards the lid of the dish) (Figure 9B).

   Note: As larval zebrafish conduct ion and oxygen exchange primarily through diffusion at this developmental stage, embedding is not detrimental.[2,3]

   a. Individuals should be arranged with adequate spacing. A minimum of 3–4 mm between individuals is sufficient which will help prevent fish losses.
   b. Agarose should be a thin layer (~2–4 mm deep) as the individuals are arranged. This requires the LMP agarose to be used to embed individuals kept at ~50–55 C. If the agar is too cool before embedding, it will solidify before individuals can be positioned.
8. After agarose completely solidifies (~3 min at room temperature(20°C–22°C)) refill the dish with E3 and move to incubator prepped for visual experience exposure (Figure 9C) (See steps 8–12).

   Note: E3 volume added should be enough to completely submerge the agar drops.

9. Mounted individuals should be placed on the dark surface in the prepped incubator beneath the light for 48 h with E3 media exchanged daily.

   Note: The agar used will be attached to the surface of the dish so E3 can be exchanged by decanting all media from the dish into a liquid waste container and completely refilling with fresh media. Dead individuals should be removed immediately using a pipette to remove the entire surrounding section of agar and dead larva.

10. Individuals should be removed from agar between 96-100 hpf. Using p10 micropipette tip or dissecting forceps, cut an outline along the individual until enough agar is removed for the larva to escape (Figure 9D).

⚠ CRITICAL: Make sure to avoid contact with the pectoral fins, jaw, and eyes to prevent damage.

11. After removal from LMP agarose, individuals should be kept at low density (~20 larvae per 6 cm dish) and in shallow E3 (15–20 mL for a 6 cm dish) to promote swim bladder inflation. Mounted individuals should be allowed a minimum of 48 h recovery before moving on to behavior testing.

Mounting zebrafish in low melting temperature agarose from 4-6 dpf

Timing: 4 days for collecting and raising larvae, 1–2 h for mounting (dependent on number of individuals mounted)

This section describes the protocol to embed larvae at a later developmental point compared to the previous major step. Larvae exposed to asymmetric visual stimulus during this period will not have an imposed sensory dependent motor bias as the critical period for this behavior has closed.

12. Complete steps 2 and 3 (See section: mounting zebrafish in low melting temperature agarose from 2-4 dpf).
13. Remove any individuals without normally inflated swim bladders.
14. By 4 dpf larvae should have naturally broken out of their chorion. The media should be thoroughly cleaned to prevent debris from occluding the mouth during mounting. Larvae can proceed to the next step at 96–100 hpf.
15. Anaesthetize individuals in 0.1% tricaine dissolved in E3 media for 1–2 min.

⚠ CRITICAL: Only individuals about to be mounted should be anaesthetized.

16. Transfer 1–5 individuals to a new dish in a small drop of E3 media.
17. Complete steps 6 and 7 (See section mounting zebrafish in low melting temperature agarose from 2-4 dpf).

Note: Larvae with swim bladders will float towards the surface of the agar. Ensure that the larvae are flat against the dish when the agar solidifies by gently pushing them back down with a p10 micropipette tip or microdissection forceps in the agar drop as it cools.

18. After agar solidifies, use a p10 micropipette tip or microdissection forceps to remove agar surrounding the mouth and head making sure not to damage the eyes or pectoral fins (Figure 9E).

Note: This is to allow for adequate oxygenation and ion exchange.

19. After agar solidifies, move mounted individuals to incubator as in step 10.
20. Exchange E3 media daily and remove dead individuals from agar as in step 10.
21. Individuals should be removed from agar at 140–144 hpf. These should be left to recover for at least 48 h following removal from agar before moving to behavior testing.
22. Following 7 dpf, larvae expend the nutrients in their yolk. Larvae should be fed with dry food in their petri dish at 7 and 8 dpf by using a micro spatula (Grainer: 21RL37) once a day.
    a. Scoop up a small amount of food (~1/32 tbsp) and dip just beneath the surface of E3 media. The dry food will disperse across the surface of the media and be adequate for feeding.
    b. Larvae should be allowed to feed for ~1 h and then food should be cleaned from the dish using a Pasteur pipette.

23. Larvae can proceed to behavior testing at 8 dpf.

Behavior testing

Timing: 30 min for set-up, 15 min per tracking series

This section outlines the behavior protocol to observe zebrafish in an open environment where external illumination is taken away. The behavior rig and software described previously (See "software setup" section) will be used to track zebrafish at 7 dpf throughout this stimulus series.

24. On the day of behavior testing, all individuals with over/under -inflated swim bladders should be removed along with any larvae that are dead or deformed (Figures 3F and 3G; Lethality, Control, n = 182, alive = 96.2%; Mounted n = 77 alive = 97.4%; Swim bladder inflation, Control n = 168 Normal inflation = 84.5%, Overinflated = 1.2%, Underinflated = 14.3%. Mounted n = 196 Normal inflation = 72.4%, Overinflated = 3.6%, Underinflated = 24.0%).

Note: Prior to behavior testing, larvae should be acclimated to the room where behavior testing will take place (~15 min). If behavior testing is taking place in a dark room, larvae should be placed on a light box with an intensity similar to standard housing conditions.

25. The behavior rig needs to be turned on and IR needs at least 15 min to warm up to ensure consistent results.
26. Petri dishes should be filled about halfway with 40 μm filtered E3 media. Filtering media can be completed with a cell strainer (Corning: 352340).

Note: Filtering removes particulate that could create noise artefacts during tracking.

27. Open up software 'EventTimer.sav' and load the following scripts (can be renamed) into separate tabs. These scripts are located at "C:/Daqtimer/scripts/".
    a. 173_explore_setparams_multiplex: Used to orient dishes and visualize contrast of behavior arena.
    b. Mp4x_t4: Used for light stimulus and tracking using a four-dish multiplexed setup.
    c. Light Test: Used to test if light turns on and off.

Note: For complete documentation on eventtimer and each program see supplemental folder (https://doi.org/10.17632/gmknc9wvwt.1) "instructions and powerpoints".

28. Align dishes to even quadrants for multiplex tracking using the grid provided in 173_explore_setparams_multiplex script. Dishes should be clear of the guidelines that separate quadrants in the camera view and edges of the camera view (Figures 10A and 10B).

Note: The camera will need to be adjusted to align with the dishes properly. The command that opens the camera (WATCH) needs to be adjusted to do this. The standard input for the watch function is found in the "Help" menu at the top of Eventtimer. WATCH: x position, x width, y position, y width, exposure, gain

In the example script, the watch function opens up a window that is 980 × 980 pixels and shifted 250 pixels in the x direction with exposure and gain set to 25.00 ms and 15 a.u., respectively.

29. Place 1 larva in each dish.
30. Adjust aperture and f-stop on lens to focus on fish and create a clear equally contrasted viewing plane. See step 22.

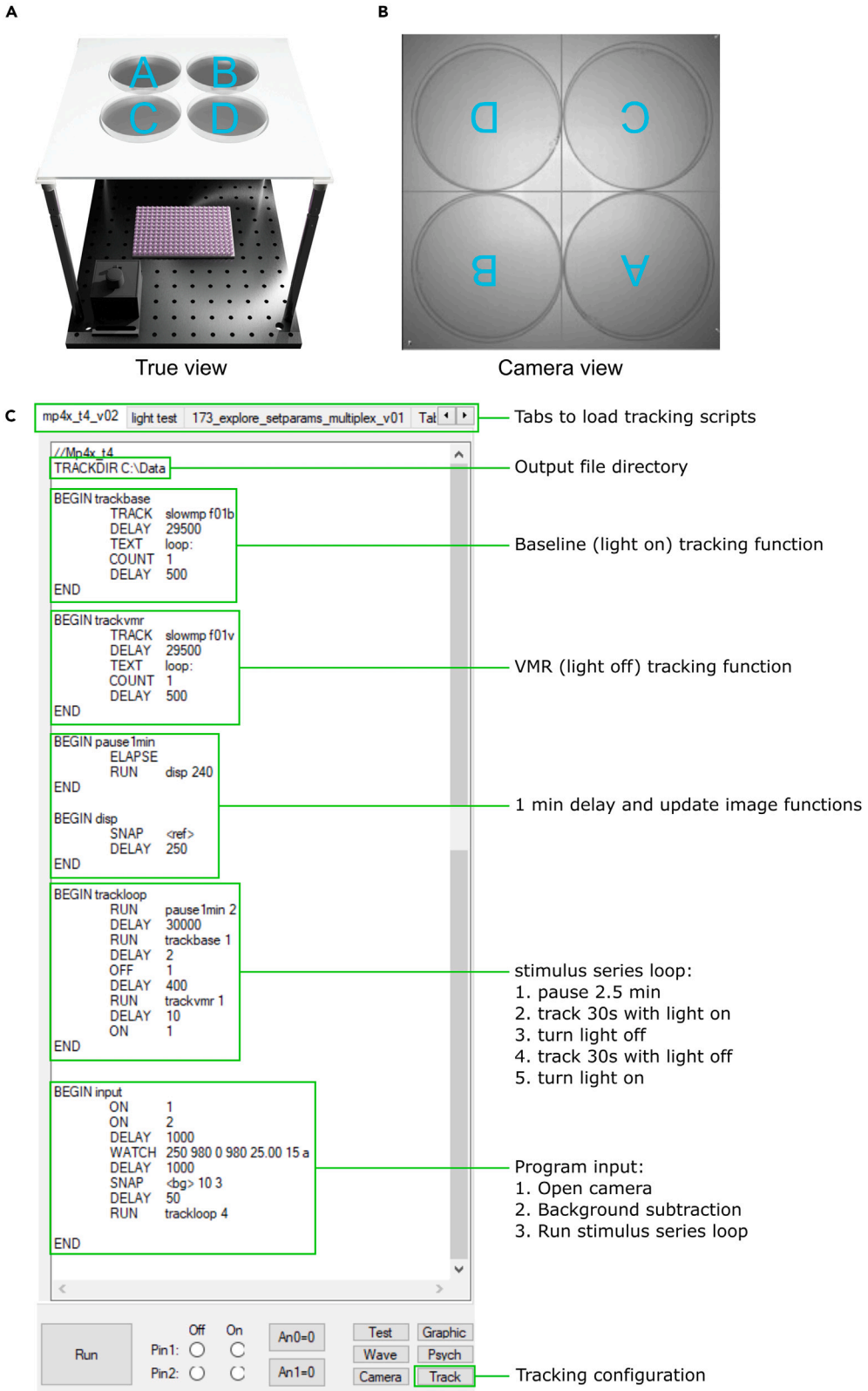

Figure 10. Behavior tracking

(A and B) (A) True perspective of dish orientation with labels used in analysis and (B) camera view for the same quadrants.
(C) Eventtimer.sav layout identifying major components of the behavior tracking script.

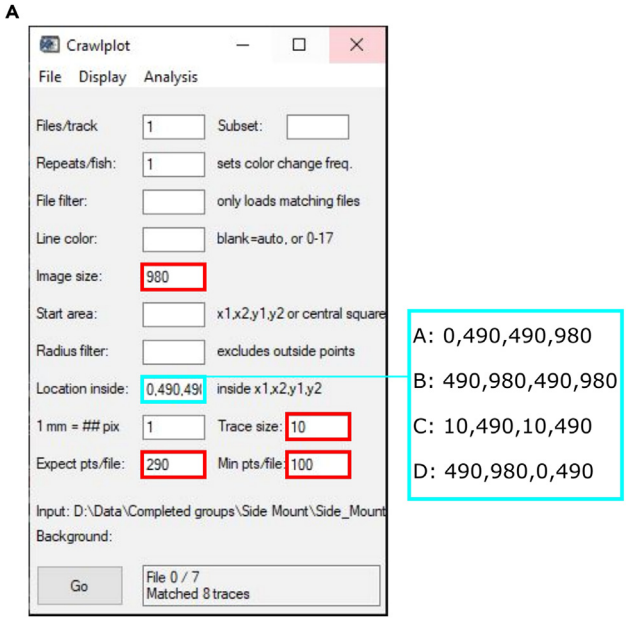

Figure 11. Cplot data analysis

(A) This is the window that appears when you open Cplot. Areas highlighted in red must use the value depicted in the figure. Image size set to 980 because the camera is opened to view a 980 × 980 pixel window when tracking larvae. Expected points per file is set to 290 because tracking 10 points per second for 29 s produces 290 points. Minimum points per file is set to 100 because tracking files where less than 100 points were acquired should be excluded. Trace size is set to 10 as this is an interpolation distance used to calculate metrics obtained later in the protocol (See step 35). Cyan inlay depicts the "Location inside" values needed to input for specific quadrants when analyzing data (See step 38).

31. Ensure light works by using the "Light Test" script. The light will turn on for 1 s, off for a second, and back on.
32. Open up "Mp4x_t4" (Figure 10C).
    a. Change TRACKDIR to the path where you would like tracking files to go.
    b. Click run and wait for console dialog to disappear.

Note: The tracking file size is ~10kb per fish (i.e., 4 fish being tracked = 40 kb).

Note: The default location for this is C:/Data/. As the tracking begins you will see the guidelines appear on the white screen denoting the four quadrants being tracked. As each individual periodically moves, a black line will be drawn to indicate their path every second. When the guidelines disappear the tracking period has ended.

33. Remove fish from dishes and repeat.

Note: For multiple runs the tracking file names will need to be changed. Each run will take 14 min to complete.

Data output

Timing: 30 min

This section describes the general process of collecting numerical output on the trajectory metrics obtained from behavior testing. For this section IDL is required where we will use the programs

cplot, and batchan to compute various trajectory metrics from tracking files obtained from behavior testing.

34. Open "Cplot" (Figure 11) and load in tracking directory using File >> Select Input
35. Set Trace Size to 10.

    Note: This is an interpolation distance for the path taken during the tracking period. Lower numbers will include more data points but be noisier while higher values will include less data points and become inaccurate.

36. Set Min pts/file to 100.

    Note: Our tracking program gathers data points for 29 s at a framerate of 10 Hz totaling 290 points. We typically exclude any tracking files with less than 100 acquired points.

37. Set image size to 980.

    Note: The "WATCH" command in the Mp4x_t4 script opens up a 980 × 980 window for tracking so this becomes the image size.

38. For each dish you will need to run a separate analysis for each quadrant, representing a single dish. Use the following parameters for the "Location inside" box for each quadrant (Figure 10A).
    a. Top left (A): 0,490,490,980.
    b. Top right (B): 490,980,490,980.
    c. Bottom left (C): 10,490,10,490.
    d. Bottom right (D): 490,980,0,490.

    Note: This is inverse to the view for tracking. See Figure 10A and 10B.

39. Click Analysis >> Output to file.
40. Click "Go" and wait for data generation to finish.
41. A new folder in your tracking directory will appear named "Analysis_cplot"
42. Within that folder is a Cplot_out file containing trajectory analysis.
    a. Append to the name the location of the dish and run analysis for the remaining positions. In our example we named these quadrants "A", "B", "C", and "D".
43. Open up Batchan.
44. Select Tools >> Reorder Rows/Cols (Figure 12A).
45. Use browser to locate the Cplot_out files and open one of them.
46. Set blocks to 8. This is the number of tracking files we have per fish. For our example this is 4 tracking periods with the light on and 4 with the light off.
47. In the dropdown menu ("Column") select CircInt.adj and click reorder.

    Note: This dropdown menu contains other metrics calculated by cplot. They are the following:

    a. Fish: Name of tracking file.
    b. Pts: Number of points gathered for a tracking period.
    c. Min. Circle: Radius of the minimum sized circle encompassing all points.
    d. ConvexHull: The convex hull of the points.
    e. Displ: Pixel distance from the first point to last point acquired.
    f. Distance: Pixel distance along the path traveled.
    g. Ellipse.Maj: Major axis of the minimum ellipse encompassing all points.
    h. Ellipse.Min: Minor axis of the minimum ellipse encompassing all points.

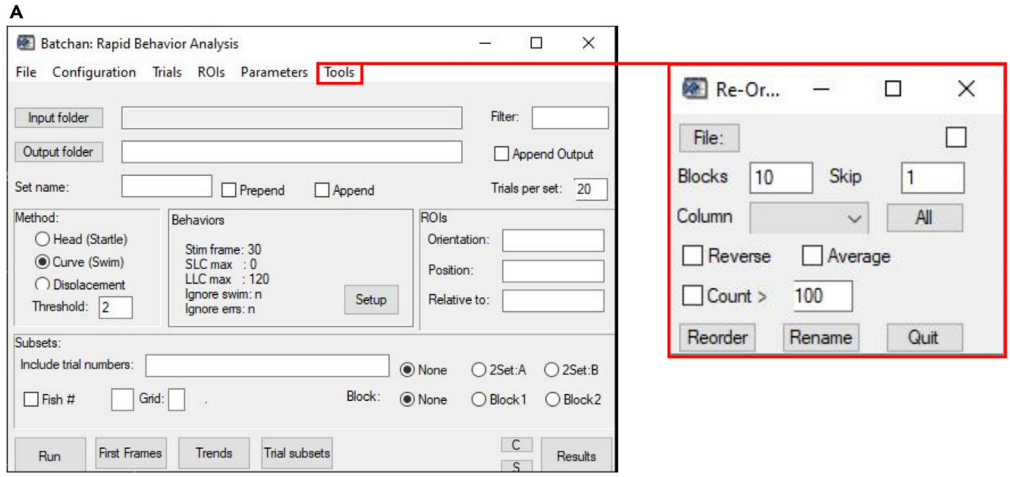

Figure 12. Batchan data analysis

(A) This is the window that appears when you open Batchan. At the top menu, select Tools then Reorder rows/cols to see the box depicted in the inlay (red).

i. qDim: Fractal dimension of the path.
j. Circ.Field: Extent of the visual field traveled. From 0-360 degrees.
k. Circ.Integ (Net turn angle): Sum of trajectory changes with respect to directionality.
l. deltaAngle.Adj (Total turn angle): Sum of absolute values of trajectory changes.
m. Max dAngle: maximum change in angle.
n. dAngle 10^{th}: top 10^{th} percentile of large turns.
Net turn angle and total turn angle nomenclature was used in the original publications.[1,4,5]

48. Repeat for DeltaAngle.adj.
49. Repeat for remaining cplot_out files.
50. Copy the data in these files to a single spreadsheet.

Note: The layout will include the name of the first tracking file as the row name followed by the values calculated for that individual in alphabetical order. The four baseline tracking values are contained in the Blk0-Blk3 columns followed by the four dark response tracking values in columns Blk4-Blk7.

51. To calculate bias ratio, divide each CircInt.adj value by the corresponding DeltaAng.adj value.
52. Average the four baseline bias ratio values together per individual along with the dark response values.

Note: The average for each embedded orientation will be negative for individuals with the right eye oriented towards the light and vice versa for individuals with the left eye oriented towards the light during the critical period. Individuals with both eyes facing the light during embedding will average to ~0.

EXPECTED OUTCOMES

This protocol is intended to provide controlled visual stimulation to zebrafish during early development, record behavior following the loss of light at 7 dpf to visualize motor bias, and observe the induction caused by asymmetric visual experience. Visual experience refines the development of visual circuits, modulates visual acuity, and can produce long-lasting changes in visual processing, which in mammalian models has been established using methods to control visual experience between the eyes.[6–12] The ability to control visual experience in zebrafish provides a model system amendable to high throughput screens and optical imaging through the entire brain volume,

providing powerful techniques to resolve mechanisms underlying visual processing, circuit formation, and impact on behavior.

LIMITATIONS

Although this method allows for robust visualization of behavior altered by the early environmental input there are several limitations. We have tested this assay by mounting individuals for 24 h or 48 h[1] but have not investigated longer durations, which may be necessary to impact behaviors other than turn bias.[1] These methods are likely not feasible post 7 dpf as we use immobilization, and as zebrafish expend their yolk by 7 dpf and need to actively hunt and feed. Conversely, earlier stage larvae (prior to 2dpf) are significantly more fragile and much more challenging to immobilize for extended periods. Also, the current software is written in the IDL virtual machine and cannot be edited without licensure for the program. However, other available tracking systems are likely to output similar metrics. Compared to other systems the method used here creates a foundation for other assays whereas systems like the Ramona Multi-Camera Array Microscope (MCAM™) and Viewpoint Visiobox™ are intended for finite uses. Our system is amenable to other behavioral assays achievable by updating and exchanging a few parts such as the camera which currently records at 10 Hz, but can be upgraded to a high-speed model.

TROUBLESHOOTING

Problem 1

Artifacts while tracking larvae (Steps 25–34).

Potential solution

Tracking artifacts are typically seen as long straight lines across the tracking field that are not due to larva movement and will distort quantitative outputs. Clear tracking arena of all reflections, which could be created by the overhead white light (typically a single location generating significant noise in the tracking). The stage and petri dishes should be dry and clear of any water drops that can reflect light and cause tracking artifacts. Reflections of water in the dish can be reduced by changing the angle of the light mount. In addition to reflections, too much or little contrast can lead to the software tracking spurious objects creating artifacts. To ensure only the larvae are tracking change tracking configuration and increase tracking threshold while decreasing "Object mass" and "Object size". Larvae need to be pigmented for tracking. Drug induced hypopigmented zebrafish will need to be allowed to develop pigment for 24 h prior to behavior testing. To minimize aberrant tracking due to contrast, ensure the larva itself is recognized for at least 80% of the tracking duration, indicated by a black line following larva movement during active tracking. Continued adjustments to tracking configurations can be made to ensure consistent tracking of larvae. Last, if tracking artifacts are occurring on the periphery of the tracking perimeter, quadrants can be further cropped to eliminate artifacts (step 38).

Problem 2

Fatal errors during data analysis (Steps 35–53).

Potential solution

Fatal errors during tracking will result in cplot to crash and close. Tracking files need to have an adequate number of points with minimal spurious artifacts. A limited number of tracking points or excess artifacts will generate a fatal error in IDL. Identify the file that cplot was analyzing prior to the crash by opening the "cplot_out" file as a spreadsheet and removing the fish from tracking files before rerunning.

Problem 3

High death rate when mounting (Steps 2–24).

Potential solution

Reduce duration of anesthetizing and ensure LMP agarose does not exceed 60°C during mounting. Also, over time LMP agarose stocks evaporate and become more concentrated, which can affect survival. Replace LMP agarose stocks regularly. Individuals who die during embedding should be removed immediately as this will help prevent further loss.

Problem 4

Visual plasticity is not working (Steps 1–97).

Potential solution

Larvae should be mounted with eyes vertically aligned. This will create a stronger asymmetry of light intensities between the eyes. Reflections in the mounting incubator may need to be reduced by adding extra neutral density filters or blackout board to the interior. In addition, any physical damage to the larvae, body or fin, while embedding larvae or removing them from agarose may impose a turning behavior and alter expected results. In this scenario, a typical indicator is observing persistent turning/looping during baseline illumination. Typically, path trajectories during baseline illumination show relatively straight movement or random directional movement.[1,4,5,13]

Problem 5

Daq module or uEye camera not functioning properly (Steps 37–45).

Potential solution

Ensure you are installing the most up-to-date drivers for both pieces of hardware. The DaqModule (USB-6001) should be functioning within NIMax and the camera should be functioning in uEye before moving to running IDL scripts. By connecting the positive end of the BNC to P0.1 on the DaqModule, you are setting the output port for IDL to port 0. If the pinout reads P1.1, you will need to change this setting in Et.sav by clicking "Configuration" and changing "Digital output" to port 1 (it is set to port 0 by default).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eric Horstick, eric.horstick@mail.wvu.edu

Materials availability

This study did not generate any new unique reagents.

Data and code availability

Original data for this protocol are available at Mendeley Data: https://doi.org/10.17632/bpj99zrshg.2. New data and software are available at Mendeley Data: https://doi.org/10.17632/gmknc9wvwt.2.

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AUTHOR CONTRIBUTIONS

J.H. wrote the manuscript, J.H. and J.S. established original protocol, A.B. and J.R.H. collected survival/morphology data, and E.J.H. supervised manuscript preparation and methodology design.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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