Video Article

Optogenetic Activation of Zebrafish Somatosensory Neurons using ChEF-tdTomato

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URL: http://www.jove.com/video/50184
DOI: doi:10.3791/50184

Keywords: Neuroscience, Issue 71, Developmental Biology, Molecular Biology, Cellular Biology, Biochemistry, Bioengineering, Anatomy, Physiology, Zebrafish, Behavior, Animal, Touch, optogenetics, channelrhodopsin, ChEF, sensory neuron, Rohon-Beard, *Danio rerio*, somatosensory, neurons, microinjection, confocal microscopy, high speed video, animal model

Date Published: 1/31/2013

Citation: Palanca, A.M.S., Sagasti, A. Optogenetic Activation of Zebrafish Somatosensory Neurons using ChEF-tdTomato. *J. Vis. Exp.* (71), e50184, doi:10.3791/50184 (2013).

Abstract

Larval zebrafish are emerging as a model for describing the development and function of simple neural circuits. Due to their external fertilization, rapid development, and translucency, zebrafish are particularly well suited for optogenetic approaches to investigate neural circuit function. In this approach, light-sensitive ion channels are expressed in specific neurons, enabling the experimenter to activate or inhibit them at will and thus assess their contribution to specific behaviors. Applying these methods in larval zebrafish is conceptually simple but requires the optimization of technical details. Here we demonstrate a procedure for expressing a channelrhodopsin variant in larval zebrafish somatosensory neurons, photo-activating single cells, and recording the resulting behaviors. By introducing a few modifications to previously established methods, this approach could be used to elicit behavioral responses from single neurons activated up to at least 4 days post-fertilization (dpf). Specifically, we created a transgene using a somatosensory neuron enhancer, *CREST3*, to drive the expression of the tagged channelrhodopsin variant, ChEF-tdTomato. Injecting this transgene into 1-cell stage embryos results in mosaic expression in somatosensory neurons, which can be imaged with confocal microscopy. Illuminating identified cells in these animals with light from a 473 nm DPSS laser, guided through a fiber optic cable, elicits behaviors that can be recorded with a high-speed video camera and analyzed quantitatively. This technique could be adapted to study behaviors elicited by activating any zebrafish neuron. Combining this approach with genetic or pharmacological perturbations will be a powerful way to investigate circuit formation and function.

Video Link

The video component of this article can be found at http://www.jove.com/video/50184/

Introduction

The development of optogenetic methods for promoting or inhibiting neuronal excitability with defined wavelengths of light has made it possible to study the function of distinct populations of neurons in neural circuits controlling behavior [1, 19, 21]. This technique is often used to activate groups of neurons, but it can also be used to activate individual neurons. Zebrafish larvae are particularly amenable to these methods since they are translucent, their nervous system develops quickly, and creating transgenic animals is fast and routine. However, significant technical hurdles must be overcome to reliably achieve single neuron activation.

To optimize a procedure for optogenetic activation of single zebrafish neurons, we focused on somatosensory neurons. Zebrafish larvae detect a variety of somatosensory stimuli using two populations of neurons: trigeminal neurons, which innervate the head, and Rohon-Beard (RB) neurons, which innervate the rest of the body. Each trigeminal and RB neuron projects a peripheral axon that branches extensively in the skin to detect stimuli and a central axon that connects to downstream neural circuits. Animals respond to touch as early as 21 hr post-fertilization (hpf), indicating that coherent somatosensory circuits have formed [5, 18]. During larval development at least some trigeminal and RB neurons synapse onto the Mauthner cell to activate classic escape responses, but accumulating evidence suggests that there are multiple classes of somatosensory neurons with different patterns of connectivity that may elicit variations on the escape behavior [2, 4, 10, 12, 14, 15, 16, 17]. Our motivation for developing this method was to characterize the behavioral function of different classes of somatosensory neurons, but this approach could in principle be used to study the function of almost any neuron or population of neurons in larval zebrafish.

Douglass *et al.* previously described a method for activating Channelrhodopsin-2-expressing somatosensory neurons with blue light, eliciting escape behavior [3]. Their approach used an enhancer element from the *isl1* gene to drive expression of ChR2-EYFP in somatosensory neurons. This transgene, however, was reported to display relatively weak fluorescence, requiring co-injection of a second reporter, *UAS::GFP*, to allow visualization of cells expressing ChR2-EYFP. This approach was used to elicit behavior responses between 24-48 hpf, but could never elicit a response past 72 hpf. Thus, while this method works for studying neural circuitry at very early larval stages (24-48 hpf), it is inadequate for characterizing neural circuits and behavioral responses in older larvae, when more diverse behavioral responses are apparent and neural circuits are more mature.

We sought to improve the sensitivity of this technique in order to characterize the function of subpopulations of larval RB neurons. To improve expression we used a somatosensory-specific enhancer (*CREST3*) [20] to drive expression of LexA-VP16 and a stretch of LexA operator sequences (4xLexAop) [11] to amplify the expression of a fluorescently tagged light-activated channel. This configuration amplified expression of the channel, eliminating the need for co-expressing a second reporter and allowing us to directly determine the relative abundance of the channel in each neuron. Using the LexA/LexAop sequence had the additional advantage of allowing us to introduce the transgene into zebrafish reporter lines that use the Gal4/UAS system. Transient expression of this transgene resulted in varying levels of expression, but was usually robust enough to visualize both the cell body and axonal projections of individual neurons over several days. To optimize sensitivity to light we used the light activated channel ChEF, a channelrhodopsin variant consisting of a chimera of channelopsin-1 (Chop1) and channelopsin-2 (Chop2) with a crossover site at helix loop E-F [13]. This channel is activated at the same wavelength as ChR2, but requires lower light intensity for activation, making it more sensitive than other commonly used channels, including ChR2. The ChEF protein was fused to the red fluorescent protein, tdTomato, enabling us to screen for protein expression without activating the channel. As a light source, we used a diode pumped solid-state (DPSS) laser coupled to a fiber optic cable to deliver a precise, high-powered pulse of blue light to a specific region of the larvae. This allowed us to focus laser light on individual neurons, eliminating the need for finding rare transgenic animals expressing the channel in a single neuron. Using this approach, we were able to activate single RB neurons, record behavioral responses with a high-speed video camera, and image the activated neurons at high resolution with confocal microscopy.

Protocol

Prepare the following ahead of time.

1. Prepare Optic Cable

1. Create a storage unit for the optic cable by melting the tapered neck of a glass Pasteur pipette over a Bunsen burner to create a ~150 ° angle.
2. Using a wire cutter or a razor blade, carefully cut the optic cable into two pieces. Each piece should have one end with a FC/PC adaptor and one exposed end. Store one piece as a reserve cable.
3. Strip the optic cable down to the cladding by removing the fiber jacket and strengthening fibers (**Figure 1A**) from 5.0 cm of cut end of the cable.
4. Insert optic cable into prepared Pasteur pipette. Make sure cable can easily move in and out of the pipette tip.
5. With optic cable protruding from tip of Pasteur pipette, carefully cut and remove fiber cladding from around glass fiber, ~2 mm from cut end.
6. Using a diamond pen/glass cutter, nick the glass fiber and break off the end to create a clean cut/surface at the end of the fiber (**Figure 1B**).
7. Retract optic cable into Pasteur pipette for storage. Repeat step (1.6) if tip of optic fiber gets chipped or breaks unevenly.
8. Position optic cable in Pasteur pipette using a micromanipulator (**Figure 1C**).

Procedures 2-8 describe a method for injecting transgenes into embryos generally applicable to many zebrafish experiments. Variations on this method, like those described in other JoVE videos [6, 7, 8, 9, 22] are equally effective.

2. Pull Injection Needles

1. Using a needle puller, pull borosilicate glass tubing into two injection needles with a gradually tapered tip. Puller settings will vary. (On a Sutter Instruments needle puller we use settings: P = 500, Heat = 720, Pull = 50, Velocity = 70 and Time = 150). Every needle puller is different, so optimize puller settings empirically. For more tapered needles, increase the Heat and/or Pull. For less tapered needles, increase Time and/or decrease Pull.
2. Store needles in a secure container (*i.e.* a Petri dish with rolled tape, adhesive side out).

3. Pour Injection Molds

1. Melt 0.5 g agarose in 30 ml embryo/blue water, until agarose has completely dissolved.
2. Pour into bottom half of a Petri dish.
3. Place rectangular mold with mounting wells (**Figure 2**) into agarose, being careful to limit bubble formation around the wells.
4. Allow agarose to solidify.
5. Remove mold and fill Petri dish with blue/embryo water.
6. Store upright, filled with clean water, at room temp for same day use or at 4 °C for future use.

4. Make Plasmid DNA Mix for Injections

1. Dilute plasmid DNA to a concentration of 50 ng/µl with 1:10 phenol red in ddH_2O. For example:

1.0 ml	plasmid DNA (250 ng/ml)
0.5 ml	phenol red
3.5 ml	ddH_2O

2. DNA mix can be kept at room temperature if using immediately or stored at 4 °C for several days.

5. Set up Mating Pairs

This should be done the evening before you plan to do injections.

1. Fill breeding tanks with system water and place male and female fish together. If injections cannot be performed as soon as lights turn on in the facility, separate male and female fish with a divider.

6. Prepare for Injections (Can be done while waiting for embryos)

1. Turn on pressure injector rig. Make sure the system is set to pulse. Start with the PULSE DURATION set to 1.
2. Turn on the AIR valve and adjust pressure injector to ~20 psi.
3. Using a dissecting scope at the highest magnification, break tip of needle with forceps or a poker to create a ~2 µm opening (**Figure 3**, **Movie 1**).
4. Fill needles with DNA mix by: 1. Placing the needle, tip side up, into the DNA mix and letting the needle fill by capillary action or 2. Using a long-reach tip to pipette 1-2 µl of the DNA mix directly into the needle.
5. Place filled needle in a safe place until ready to inject.

7. Collect Embryos

1. After facility lights have turned on, remove dividers (if applicable). Collect embryos with a strainer/small sieve and transfer them to a Petri dish with fresh blue/embryo water.

8. Inject Embryos at the 1-2 Cell Stage

1. Transfer harvested embryos to injection molds using a plastic pipette.
2. Using a dissecting microscope, gently push embryos into wells with forceps or a blunt poker (**Figure 4**, **Movie 2**).
3. Place loaded needle into a micromanipulator and position over embryos.
4. Calibrate injection volume by adjusting the PULSE DURATION in 1-step increments until you achieve the desired volume (~1 nl). This could be calibrated with a micrometer slide, as described in a previous JoVE videos [8, 22], but for this experiment precision is not necessary, since a wide range of injection volumes will result in adequate expression.
5. Inject ~1 nl DNA mix directly into the cell and repeat for all embryos. DNA can also be injected into the yolk, but this tends to be less efficient (**Figure 5**, **Movie 3**).
6. Remove injected embryos from mold using a gentle stream of blue/embryo water.
7. Store injected embryos at 28.5 °C in the dark.
8. Remove unfertilized, damaged or dead embryos periodically.
9. Treat embryos with PTU between 18-24 hpf to prevent pigmentation.

9. Screen for Transgene Expression

1. Manually dechorionate 24-48 hpf embryos using forceps.
2. Anesthetize larvae using 0.02% tricaine.
3. Using a fluorescent dissecting microscope, identify larvae with RB or trigeminal neuron expression and transfer these to a new dish with fresh PTU blue/embryo water. Embryos with sparse expression in easily identifiable cells are optimal, but individual neurons will be targeted for activation with a fiber optic cable so a wide range of expression density is acceptable.
4. Store embryos at 28.5 °C in the dark until desired experimental stage.

10. Mount Larvae for Behavior Experiments

1. Make 1.5% low melt agarose in ddH_2O and store in a 42 °C heat block to prevent it from solidifying.
2. Using a glass Pasteur pipette, transfer one of the pre-screened larvae into a tube of 1.5% low melt agarose with as little blue/embryo water as possible.
3. Transfer larvae in a drop of agarose onto a small Petri dish.
4. Under a dissecting microscope, position larva dorsal up.
5. When agarose has solidified, cut away the agarose with a thin razor blade (#11 scalpel), leaving a wedge of agar around the entire larva.
6. Make two diagonal cuts at either side of the yolk; take care not to nick the larva.
7. Fill the area surrounding the agarose with embryo/blue water.
8. Pull agarose away from the trunk and tail of the larva (**Figure 6**).

11. Prepare High-speed Camera and Imaging Software

1. Mount high-speed camera onto dissecting scope.
2. Connect camera to computer.
3. Turn on computer.
4. Turn on high-speed camera.

5. Open video/imaging software (We use AOS imaging software and will describe procedures for using it here, but other imaging software is equally acceptable).
6. Adjust camera settings accordingly (*i.e.* 1,000 frames per sec (fps), 50% trigger buffer or other preferred settings).
7. Start recording.

12. Activate Single Neurons Using a 473 nm Laser

1. Attach stimulator, laser and optic cable.
2. Turn on stimulator.
3. Set stimulator to a maximum of 5 Volts and a pulse duration of 5 msec.
4. Turn on laser according to manufacturer's instructions.
5. Use a dissecting microscope to position the tip of the optic cable near cell body of a neuron with ChEF-tdTomato expression (**Figure 6**).
6. Deliver pulse of blue light to activate sensory neuron.
7. Record behavior using a high-speed camera set at 500 or 1,000 frames per sec.
8. Repeat experiment as desired, waiting 1 min between each activation to avoid habituation. (We record a minimum of three responses for each neuron).
9. To release larvae, pry apart agarose with forceps, taking care not to injure the animal. This animal can be allowed to develop further and the procedure can be repeated at an older stage to characterize development of the behavior. The embryo can also be remounted for high-resolution confocal imaging of the activated cell to correlate behavior with cellular structure, as described below.
10. Transfer larva to culture plate with fresh blue/embryo water. We use a 24-well plate to keep track of individual larvae.

13. Image Neuron(s) with a Confocal Microscope

1. Anesthetize larvae with 0.02% tricaine.
2. Mount larvae, dorsal side up, in 1.2% low melt agarose or in a dorsal mold.
3. Image ChEF-tdTomato neurons with a 543 nm laser and appropriate filter and objective. We use a 20x objective.
4. Remove larvae from agarose and return to individual well with blue/embryo water.
5. Store at 28.5 °C in the dark for further analysis.

Representative Results

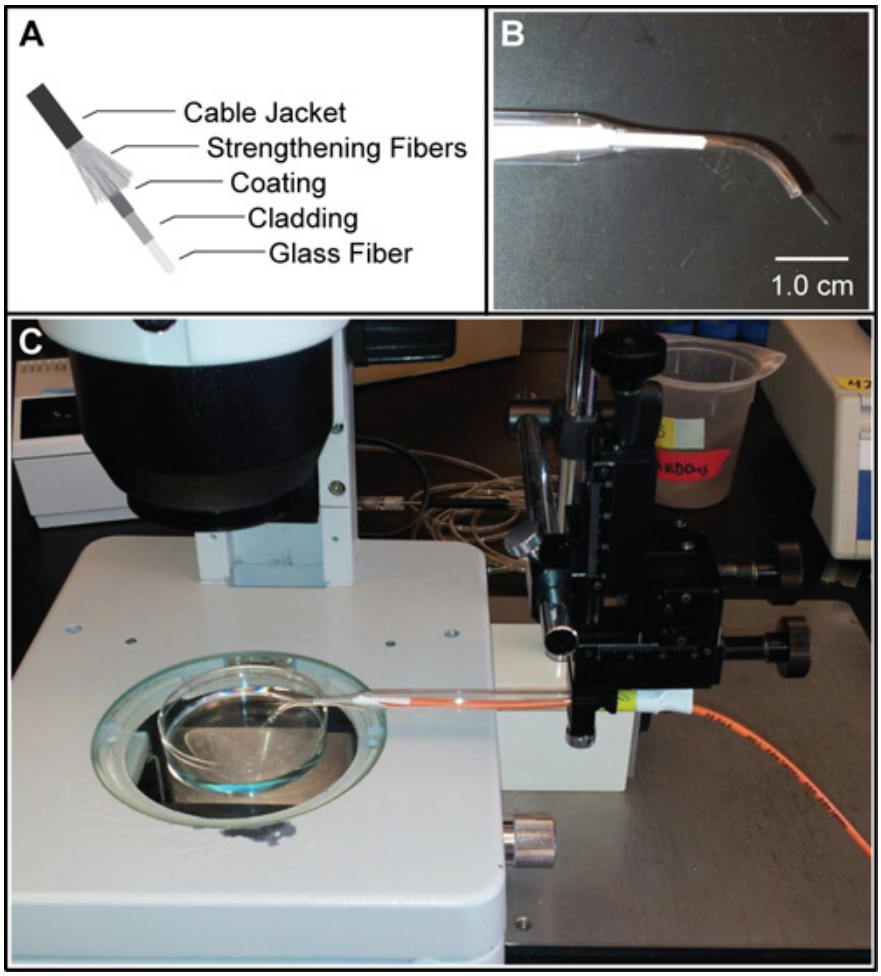

Figure 1. Optic cable set up. (A) Layers of a fiber optic cable. (B) Stripped fiber optic cable in a Pasteur pipette. (C) Fiber optic cable in Pasteur pipette positioned using a micromanipulator.

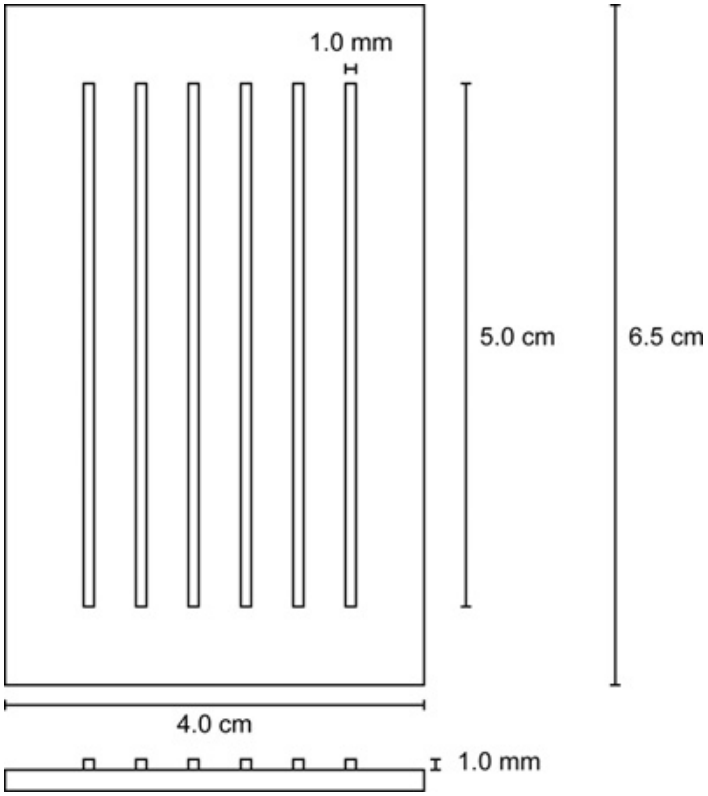

Figure 2. Injection mold template.

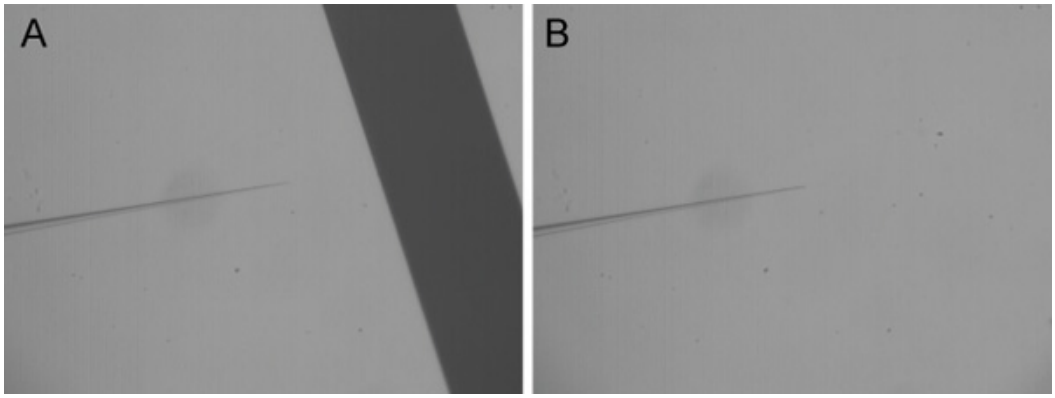

Figure 3. (Movie 1). Breaking the needle.

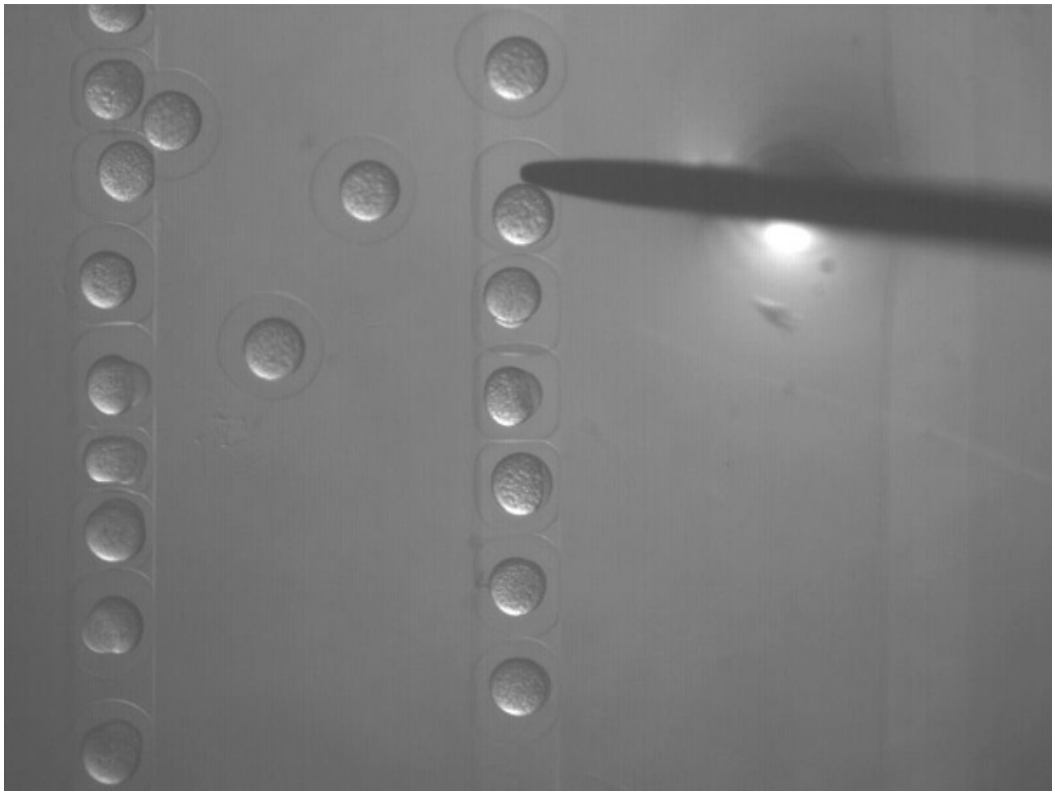

Figure 4. (Movie 2). Placing embryos into the injection mold.

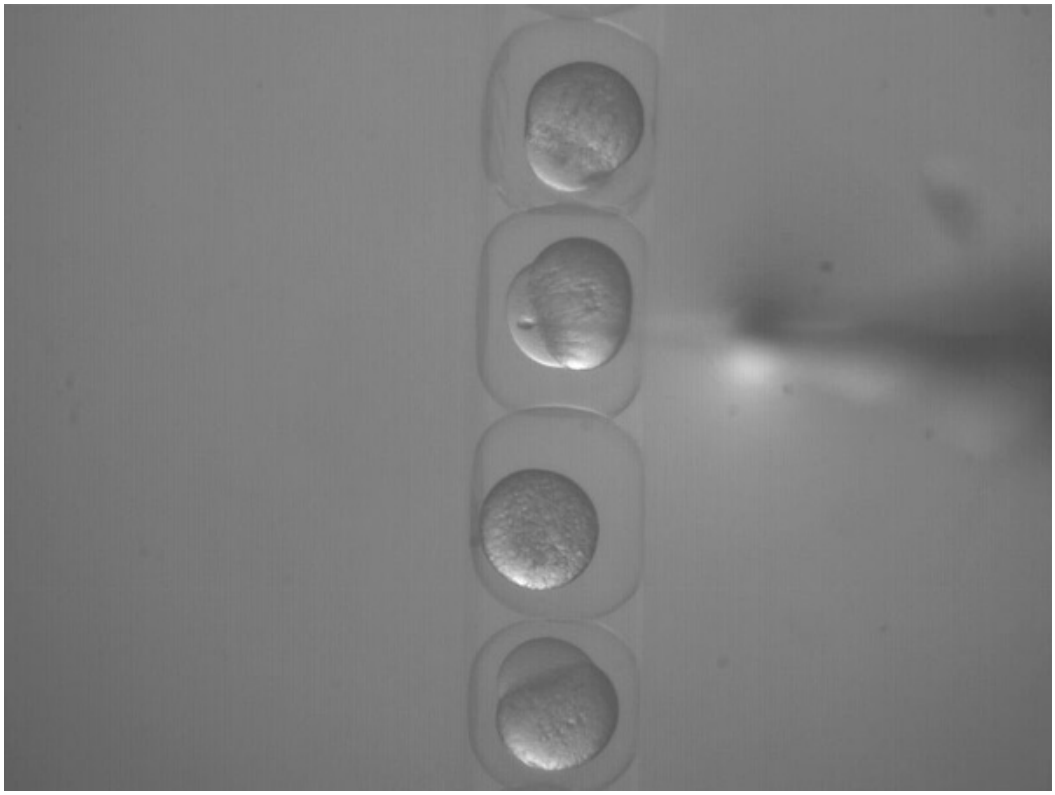

Figure 5. (Movie 3). Injecting 1 nl DNA mix into 1-cell stage embryo.

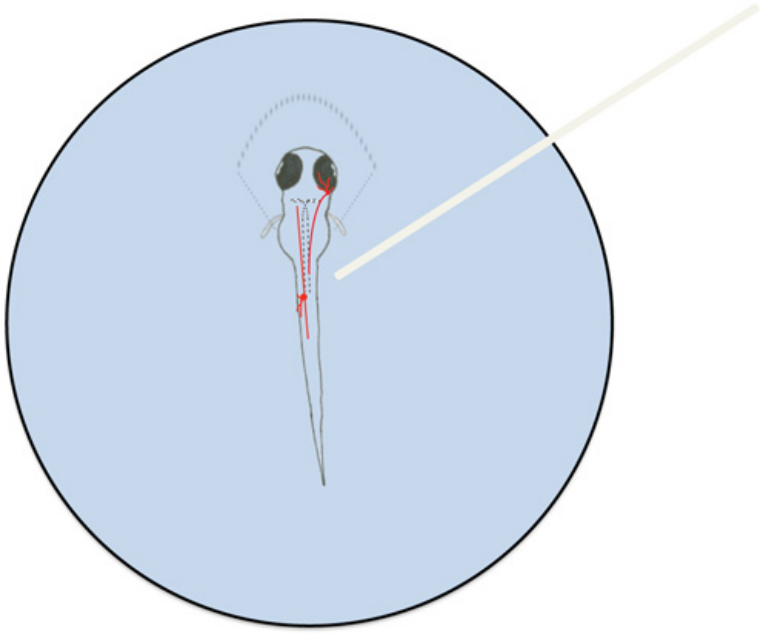

Figure 6. Diagram of a mounted zebrafish larva and representative neurons involved in the larval touch response. Zebrafish larvae were partially mounted in 1.5% low melt agarose (represented by dashed lines surrounding the rostral portion of the larva). A trigeminal neuron (in the head) and a Rohon-Beard neuron (in the trunk) are depicted in red. Mauthner cells are outlined by dashed lines in the larva. The optic cable (white) is shown positioned over the RB neuron cell body.

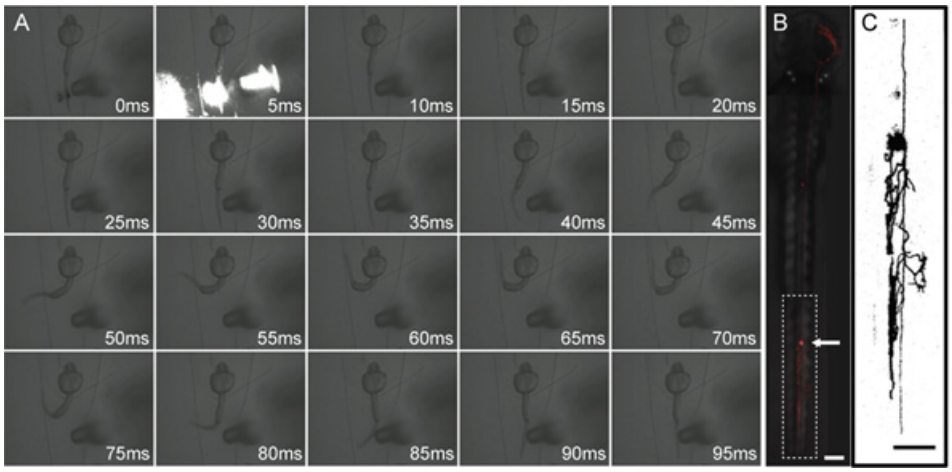

Figure 7. (Movie 4).Activation of a single RB neuron expressingChEF elicits a behavioral response. (A) Still frames depicting activation of a single RB neuron expressing ChEF-tdTomato. Single neurons were activated by a 5 msec pulse from a 473 nm blue laser *via* a 200 µm fiber optic cable. Voltage driving the blue laser was set at a maximum of 5 volts. The resulting behavior was recorded at 1,000 frames per sec by a high-speed camera. (B) Confocal microscopy was used to image the activated RB neuron. Arrow shows region stimulated in behavior stills. (C) Magnified image of RB neuron indicated in (B). Scale bar, 100 µm. (**Movie 5** and **6**) same fish, (**Movie 5**) trigeminal activation, (**Movie 6**) RB neuron over yolk extension. Click here to view larger figure.

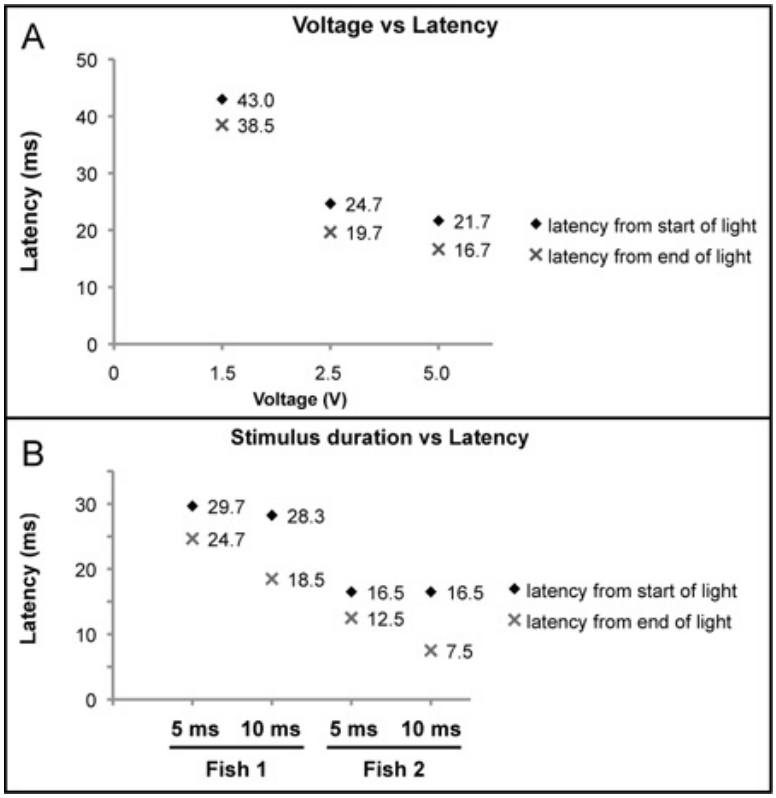

Figure 8. Latency of behavior under variable conditions. For most experiments we activated a single neuron in ~35 hpf larvae expressing ChEF-tdTomato with a 5 msec pulse from a 473 nm blue laser driven by a 5 V power source (**Figure 6**). To better understand the dynamics of ChEF activation, we varied parameters to determine their effect on behavior. (A) The duration of light stimulation (5 msec versus 10 msec) did not affect latency when quantified from start of light pulse. (B) At ~35 hpf, voltage inversely affects latency. Lower voltages resulted in an increase in latency of movement. For our experiments, we used the maximum voltage (5 V) permissible for our laser apparatus, which elicited reliably stereotyped behaviors from most brightly-expressing RB neurons.

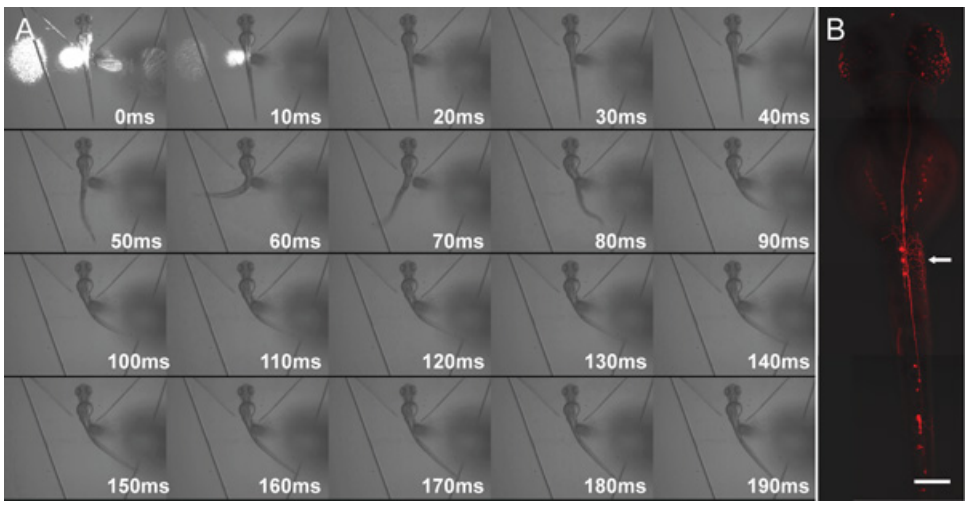

Figure 9. (Movie 7). Activation of ChEF-tdTomato expressing RB neurons in 60 hpf larvae. (A) Still frames depicting activation of RB neurons expressing ChEF-tdTomato by a 10 msec pulse from a 473 nm blue laser *via* a 200 µm fiber optic cable. The resulting behavior was recorded at 1,000 frames per sec by a high-speed camera. (B) Confocal microscopy was used to image the activated RB neuron(s). Arrow shows region stimulated in behavior stills. Scale bar, 100 µm.

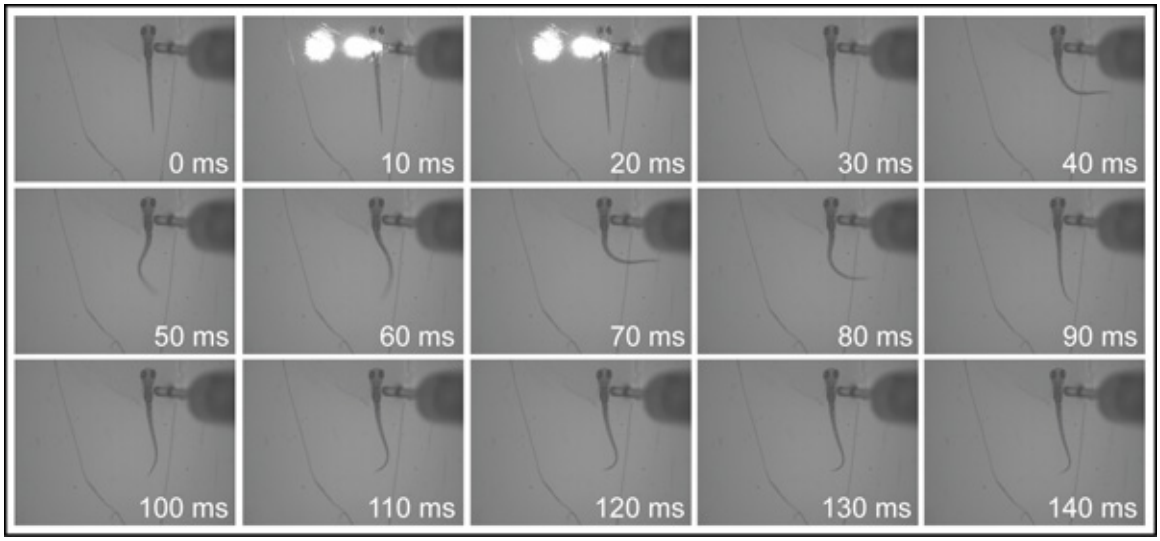

Figure 10. (Movie 8). Activation of ChEF-tdTomato expressing RB neurons in 4 dpf larvae. Still frames depicting activation of RB neurons expressing ChEF-tdTomato by a 20 msec pulse from a 473 nm blue laser *via* a 200 µm fiber optic cable. The resulting behavior was recorded at 1,000 frames per sec with a high-speed camera.

Movie 1. Click here to view movie.

Movie 2. Click here to view movie.

Movie 3. Click here to view movie.

Movie 4. Click here to view movie.

Movie 5. Click here to view movie.

Movie 6. Click here to view movie.

Movie 7. Click here to view movie.

Movie 8. Click here to view movie.

Discussion

We have described an approach for optogenetic activation of single RB neurons in live zebrafish. Our method employs transient transgenesis to express a fluorescently tagged channelrhodopsin variant, ChEF-tdTomato[13], in specific somatosensory neurons. This approach could easily be adapted for use in other larval zebrafish cell populations.

Using this approach we consistently elicited behavioral responses from 34-48 hpf larvae expressing ChEF-tdTomato. Using a 5 msec pulse of blue light at 5 V, we were able to activate single RB neurons (**Figure 7**). By positioning the optic fiber at different points along the animal, we found that it was necessary to aim the blue light directly at a cell body to elicit a behavioral response. Light pulses did not elicit a response from larvae that did not express ChEF. In addition, light pulses along the central axon or the peripheral axon never elicited a response, even in young larvae (data not shown). This property was advantageous since we could confidently activate single neurons in animals in which multiple neurons were labeled, even if the central axons of other neurons passed near the targeted neuron's cell body (**Movie 4-6**).

To test the reliability of the approach for assessing kinematic parameters, we determined the latency of the escape response (the time from light activation to behavioral response) between 40 and 48 hpf, a parameter that is known to be highly stereotyped. To determine if the duration of light stimulus influences neuron activation, we illuminated target neurons for 5 or 10 msec (**Figure 8A**). Since the behavioral latency in both conditions were the same when calculated from the start of the light pulse, we concluded that the duration of the light pulse did not influence latency of behavior. However, we did find that reducing the voltage increased the latency of a behavior (**Figure 8B**). At 5 V, however, many responses (9 out of 16 fish) were 20 ± 5 msec, similar to the latency reported for natural escape responses. Thus, activating neurons with 5 V approaches maximal activation.

Parameters for effectively eliciting behavioral responses from activating single RB neurons varied in older larvae. We successfully elicited behavioral responses from animals as old as 4 dpf, substantially later than was previously reported. However, while activation of single RB neurons with a 5 msec light pulse at 5 V consistently resulted in a behavioral response before 48 hpf, activation of older larvae (>60 hpf) was not as consistent. Longer pulses (10-100 msec) of light were often necessary to activate neurons in older larvae (**Figure 9** and **10**, **Movie 7** and **8**, respectively). Therefore, activation parameters may need to be optimized/calibrated based on experimental stage.

The approach we describe here could be used for many potential applications. We are using this method to define the behavioral responses elicited by different subtypes of neurons, but it could also be used to characterize the development of behavioral responses as the animal

matures, the effects of drugs or mutants on behavior, or, in combination with physiology or imaging, to characterize downstream circuits activated by an identified neuron. Conversely, inhibitory channels, such as halorhodopsin, could be used to inhibit specific neurons within a neural network.

Disclosures

The authors declare that they have no competing financial interest.

Acknowledgements

We thank Fumi Kubo, Tod Thiele and HerwigBaier (UCSF/Max Planck Institute) for advice on behavior experiments and DPSS laser set up; Heesoo Kim and Chiara Cerri from the MBL Neurobiology Course for assisting in ChEF-tdTomato experiments; PetronellaKettunen (University of Gothenburg)for initial collaboration on optogenetic experiments; BaljitKhakh, Eric Hudson, Mike Baca and John Milligan (UCLA) for technical advice; and Roger Tsien (UCSD) for the ChEF-tdTomato construct. This work was supported by an NRSA (5F31NS064817) award to AMSP and a grant from the NSF (RIG:0819010) to AS.

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