Video Article

Spinal Cord Transection in the Larval Zebrafish

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Abstract

Mammals fail in sensory and motor recovery following spinal cord injury due to lack of axonal regrowth below the level of injury as well as an inability to reinitiate spinal neurogenesis. However, some anamniotes including the zebrafish *Danio rerio* exhibit both sensory and functional recovery even after complete transection of the spinal cord. The adult zebrafish is an established model organism for studying regeneration following spinal cord injury, with sensory and motor recovery by 6 weeks post-injury. To take advantage of *in vivo* analysis of the regenerative process available in the transparent larval zebrafish as well as genetic tools not accessible in the adult, we use the larval zebrafish to study regeneration after spinal cord transection. Here we demonstrate a method for reproducibly and verifiably transecting the larval spinal cord. After transection, our data shows sensory recovery beginning at 2 days post-injury (dpi), with the C-bend movement detectable by 3 dpi and resumption of free swimming by 5 dpi. Thus we propose the larval zebrafish as a companion tool to the adult zebrafish for the study of recovery after spinal cord injury.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51479/>

Introduction

Major trauma to the human spinal cord often results in permanent paralysis and loss of sensation below the level of injury, due to the inability to regrow axons or reinitiate neurogenesis^{1,2}. In contrast to mammals, however, anamniotes including salamanders and zebrafish (*Danio rerio*) show robust recovery even after complete spinal cord transection^{3,4}.

The adult zebrafish is a well-established model for studying the recovery process following spinal cord injury⁵⁻⁷. Following complete spinal cord transection, reestablishment of sensory and locomotive function is observed in the adult zebrafish by 6 weeks post-injury⁸. In order to examine the regenerative process *in vivo*, we turned to the transparent larval zebrafish⁹.

Here we present a method to transect the spinal cord of a 5 days post-fertilization (dpf) larval zebrafish using a beveled microinjection pipette as a scalpel, modified from Bhatt, *et al.*¹⁰ This method supports high throughput, low mortality, and reproducibility. With practice, 300 larvae/hr can be transected, and over 6 months of transections, including over 3,600 animals, 98.75% $\pm$ 0.72% survived until 7 days post-injury (dpi). Our data shows rapid recovery of sensory and locomotion as well: at 1 dpi, all movement by the injured fish is driven by pectoral fin locomotion only. However, larvae begin to respond to tungsten needle touch caudal to transection by 2 dpi, reestablish C-bend movement by 3 dpi, and display predatory swimming by 5 dpi¹¹. Using antibody staining against acetylated tubulin, we have confirmed that axons are absent from the injury site at 1 dpi, but have crossed the injury site by 5 dpi. We believe this protocol will provide a valuable technique for the study of axonal regrowth and neurogenesis in the spinal cord following injury.

Protocol

Zebrafish were raised and bred according to standard procedures; experiments were approved by the University of Utah Institutional Animal Care and Use Committee.

1. Preparation of Surgery Plates

1. Make surgery plates using 60 mm Petri dishes and Sylgard 184 Silicone Elastomer Kit, following manufacturer's instructions. Fill dishes no more than half-full and allow to polymerize. Store covered at room temperature.

2. Preparation of Micropipettes

1. Fabricate micropipettes by heating and pulling thin-wall borosilicate capillary tubing in a micropipette puller using the same settings for making microinjection needles.

2. Under a dissection microscope, snap off tip of micropipette to approximately 200 μm in diameter with forceps.
3. Bevel broken edge with a microgrinder initially to 35°, followed by a second beveling at 25°. Ensure tip is sharp and smooth. Store finished beveled micropipette in a Petri dish on a small amount of clay.

3. Preparation of Zebrafish Larvae

1. 7 days prior to surgery, set up mating tanks of male and female zebrafish.
2. Collect embryos the following morning, 3 hr after the lights come on to ensure maximum yield. If using a transgenic reporter line such as *Tg(elevl3:eGFP)^{knu3}*, sort fertilized embryos 100/100 mm plate in 25 ml of E3 at 28.5 °C. If using wildtype, sort fertilized embryos 25/100 mm plate in 25 ml of E3 at 28.5 °C.
3. If using a reporter line, screen embryos for fluorescent expression at 48 hpf. Allow identified embryos to mature at a density of 25/100 mm plate in 25 ml of E3 at 28.5 °C.
4. When larvae are 5 dpf, prepare surgery plate by covering Sylgard with E2 + 10 mg/L Gentamycin Sulfate (GS) + Tricaine.
 1. Prepare recovery dish by adding 25 ml E2 + GS to a 100 mm Petri dish.
 2. Prepare scalpel by taping together three swabs. This will form a triangular tool with three grooves.
 3. Mount a prepared micropipette on the swabs by taping it into one of the grooves.
5. If reusing micropipettes, flush until clear with E2 + GS using a 1ml syringe and a 27 G needle prior to mounting on swabs.

4. Surgery

1. Anesthetize 1 plate of larvae at a time (25 fish) with Tricaine. Fish are sufficiently anesthetized when they no longer exhibit touch response. It is important that fish are completely anesthetized prior to surgery, otherwise they will twitch when the scalpel touches them. Surgery is performed under a dissection microscope.
2. Transfer larvae to surgery plate.
 1. Under maximum magnification, rotate one larva at a time so that it lies on its side with its back closest to the hand holding the scalpel.
 2. Position forceps so that they rest on the Sylgard, angled over the width of the larva.
 3. Bracing the glass scalpel against one of the arms of the forceps, cut into the dorsal lateral face of the larva at the level of the anal pore, being sure not to cut beyond the ventral edge of the notochord. Twist the scalpel to sever the spinal cord.
 4. Repeat with remaining larvae.
Note: if a larva bleeds, it will not recover from the surgery. Immediately remove the larva from the surgery plate and euthanize it via Tricaine overdose.
3. Once surgery on the batch of larvae is complete, transfer injured animals to the recovery plate. This is to support the clearing of anesthesia.
 1. Caution: when collecting injured larvae for transfer, make sure they are collected head or tail first: do not stress the injury site by bending the larvae.
Note: All devices used for surgery can be reused, including the micropipettes.

5. Recovery

1. Transfer injured larvae from the recovery plate to 100 mm plates filled with 25 ml E2 + GS at a density of 25/plate. Allow to recover in a 28.5 °C incubator.
2. Check plates daily, removing sick and dead animals. Do not change the media until *Coleps* (freshwater protozoa) are visible in the media. When changing the media, do not transfer the fish to a new plate; instead, remove as much media as possible and flood the same plate with new media. Repeat as necessary to reduce *Coleps* population.
3. Feed daily with a small amount of powdered fry food.
Note: Live food (e.g., paramecia or rotifers) cannot be fed to injured larvae until after they have recovered locomotion. Otherwise, the live food will colonize the injury site and kill the larvae.

Representative Results

To reduce severity of tissue damage surrounding the injury site, proper beveling of the micropipette is critical. **Figure 1A** shows a correctly beveled tip. Using a tip that is too wide (**Figure 1B**) tends to result in higher fatalities due to the increased likelihood of nicking the dorsal aorta, while a tip that is too narrow (**Figure 1C**) tends to glance off the skin rather than cutting tissue.

To practice this technique, it is advantageous to use a reporter line such as *Tg(elevl3:eGFP)^{knu3}* to visualize the spinal cord. **Figure 2A** shows a completely transected spinal cord of a live *Tg(elevl3:eGFP)* zebrafish at 1 dpi, while **Figure 2B** shows the same live fish at 3dpi. **Figures 2C** and **2D** show higher magnifications of the injury site at 3 dpi in fixed *Tg(dbx1a:eGFP)* fish having complete (**Figure 2C**) or incomplete (**Figure 2D**) spinal cord transection. Note the contiguous region of neuron labeling along the ventral edge of the spinal cord (yellow arrow).

Figure 1. Comparison of scalpel edges. **A** shows a correctly beveled micropipette tip suitable for surgery. This size is readily cleaned for reuse. **B** shows a beveled micropipette tip too wide for surgery on a 5 dpf larva. **C** is an example of a tip that is too narrow. This size is very difficult to clean for reuse, and tends to promote a sawing action of transection instead of cutting. **D**: cartoon of the lesioning tool assembly. Three 6" swabs are nested into a pyramidal shape and taped together. The scalpel rests in one of the grooves formed by the three swabs, and is taped in place.

Figure 2. Verifying complete transection. Fluorescent confocal microscopy was used to image live *Tg(elavl3:eGFP)* fish *in vivo* at 1 dpi (A) and 3dpi (B). To confirm complete transection, these image stacks were then processed in ImageJ (rsbweb.nih.gov) to generate Maximum Intensity Projections (MaxZ) as shown in A-B. C-D show MaxZ projections of HuC/D labeled *Tg(dbx1a:eGFP)* fish at 3 dpi with complete spinal transection (C) or incomplete transection (D). Yellow arrows identify injury site, D=dorsal, R=rostral. Scale bar = 100 μm.

Discussion

When initially learning this technique, we recommend attempting no more than 50-100 transections in a single session. After mastering this technique, we are able to transect up to 300 embryos per hr; however, this level of throughput requires a few months of weekly practice. We also recommend practicing with a reporter line and verifying complete transection until the incidence of incomplete spinal cord transection is reduced to less than 1%.

Spinal cord transection in the adult zebrafish is a well-established and robust technique for studying axonal regrowth and neurogenesis after injury. By moving this analysis into the larval organism, we are able to examine recovery *in vivo*. Additionally, we are also able to utilize genetic tools not available in the adult zebrafish to examine the roles of various genes in the regenerative process, *e.g.*, *Tcf7l1a*¹².

Originally developed to study neurogenesis following spinal cord transection, this technique can also be used to examine recovery of sensory function: injured animals show a response to touch caudal to the injury site by 2 dpi, and axons have crossed the injury site by 5 dpi.

Disclosures

The authors have nothing to disclose.

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