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METHOD

A Surgery Protocol for Adult Zebrafish Spinal Cord Injury

Ping Fang, Jin-Fei Lin, Hong-Chao Pan, Yan-Qin Shen*, Melitta Schachner*

Center for Neuroscience, Shantou University Medical College, 22 Xinling Road, Shantou 515041, China

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ABSTRACT

Adult zebrafish has a remarkable capability to recover from spinal cord injury, providing an excellent model for studying neuroregeneration. Here we list equipment and reagents, and give a detailed protocol for complete transection of the adult zebrafish spinal cord. In this protocol, potential problems and their solutions are described so that the zebrafish spinal cord injury model can be more easily and reproducibly performed. In addition, two assessments are introduced to monitor the success of the surgery and functional recovery: one test to assess free swimming capability and the other test to assess extent of neuroregeneration by *in vivo* anterograde axonal tracing. In the swimming behavior test, successful complete spinal cord transection is monitored by the inability of zebrafish to swim freely for 1 week after spinal cord injury, followed by the gradual reacquisition of full locomotor ability within 6 weeks after injury. As a morphometric correlate, anterograde axonal tracing allows the investigator to monitor the ability of regenerated axons to cross the lesion site and increasingly extend into the gray and white matter with time after injury, confirming functional recovery. This zebrafish model provides a paradigm for recovery from spinal cord injury, enabling the identification of pathways and components of neuroregeneration.

KEYWORDS: Zebrafish; Spinal cord injury; Surgery; Locomotor recovery; Axonal regrowth

1. INTRODUCTION

Spinal cord injury (SCI) is a neurological problem that causes severe suffering and disability in patients. Currently, no effective therapy exists. Experimental animal models have been invaluable in increasing our knowledge of the molecular bases underlying the inability of mammals to recover from SCI. Such animal models include monkeys, rats and mice subjected to surgical injury, involving compression lesion, hemisection and complete transection (Mehanna et al., 2010; Nout et al., 2012; Sakai et al., 2012). Adult zebrafish retains the ability to regain a high level of neurogenesis, neuritogenesis and formation of functional synapses, and maintain low levels of apoptosis in response to nerve injury (Hui et al., 2010; Kroehne et al., 2011). Zebrafish thus has been adopted as a model to study recovery from SCI due to their extraordinary ability to regenerate successfully after central nervous system trauma. Axonal regrowth in adult zebrafish achieves restoration of function by approximately 4−6 weeks after complete spinal cord transection (Becker et al., 1998; van Raamsdonk et al., 1998a, 1998b; Becker and Becker, 2001; Fawcett, 2006; Reimer et al., 2008; Yu et al., 2011b). The adult zebrafish injury and regeneration model should be useful for identification of beneficial genes and permissive epigenetic modes of gene regulation, and thus pave the way to gain insight into therapeutic venues for humans.

Although the zebrafish model has been used for many years, the specific procedure for spinal cord transection, which can be easily performed, has not been described in detail. Here, we give a step-by-step surgical protocol to perform SCI in adult zebrafish. In addition, we describe two methods to evaluate the success of the zebrafish SCI model and the restoration of locomotor ability: a freely swimming behavioral analysis and anterograde axonal tracing *in vivo* (Becker et al., 2004; Guo et al., 2011; Yu et al., 2011a). Swimming distance is measured each week after SCI until the 6th week (Becker

* Corresponding authors. Tel: +86 754 8890 0276, fax: +86 754 8890 0236.
E-mail addresses: yqshen@stu.edu.cn, shenyq30@hotmail.com (Y.-Q. Shen); schachner@stu.edu.cn (M. Schachner).

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et al., 2004; Guo et al., 2011). To trace regenerating axons past the lesion site, biocytin is applied at the brainstem/spinal cord junction so as to be internalized by severed axons and anterogradely transported to their synapses (Becker and Becker, 2001; Yu et al., 2011a, 2011b). Axons labeled by biocytin permit the evaluation of their potential to regrow across the lesion site by visualizing the restoration of contacts distal to the lesion site through the tracing of axons projecting from various nuclei in the brain to their targets.

2. MATERIALS AND METHODS

2.1. Reagents

- Phosphate-buffered saline, pH 7.4 (PBS)
- Tricaine solution: 0.03% ethyl 3-aminobenzoate methanesulfonate (MS222, Sigma, St. Louis, MO, USA) in PBS
- 75% ethanol in distilled water (v/v)
- Zebrafish food (QianHu, Singapore, Singapore)
- Anti-fungal agents (Maroxy, Omaha, NE, USA)
- Tissue-Tek O.C.T. (Optimal Cutting Temperature) Compound (Sakura Finetek, Torrance, CA, USA)
- 4% paraformaldehyde in PBS (w/v)
- 15% sucrose in PBS (w/v)
- Biocytin (Sigma, St. Louis, MO, USA)
- Streptavidin-Cy3 in PBS (1:200 v/v, Bioss, Beijing, China)
- DAPI in PBS (1:5 v/v, Beyotime, Shanghai, China)
- Gelfoam (Upjohn, Kalamazoo, MI, USA)
- Histoacryl (B. Braun, Melsungen, Germany)
- Crushed Ice

2.2. Instruments

- Spring scissors (15000-00, 15000-03, 15000-08, Fine Science Tools, Inc. Heidelberg, Germany) (Fig. 1A)
- Forceps (11274-20, 11254-20, Fine Science Tools, Inc. Heidelberg, Germany) (Fig. 1A)
- Video camera (Aidosens, Guangzhou, China)
- Tank (42 cm × 30 cm × 30 cm, Animal Center of Shantou University Medical College, Shantou, China)
- Stereomicroscope (JW-101, OKA, Wuzhou, Guangxi, China) (Fig. 1B)

2.3. Animals

Adult zebrafish (*Danio rerio*) should be about 6 months old and 2.5−3 cm in length, although this could vary depending on the health status of the fish and how well they have been fed. The health status is important for the success of the surgery and batches of fish that do not recover well should not be used for further experiments. Attempts to improve the health status of the fish by maintenance with anti-fungal solution and antibiotics have not been successful. Our fish were purchased from the Huiyuan Aquatic Animals Company (Shantou, Guangdong, China) and maintained on a 14 h light and 10 h dark cycle at 28°C. The fish water contains anti-fungal agents, and should be kept constant at pH 7.4. Lights come on in the fish room at 8 am. Fish were fed with zebrafish food, described in the reagents list, twice a day. Either males or females can be used for sham surgery or SCI. The number of zebrafish needs to be calculated before the start of the experiment, taking into consideration an approximate 70%−80% of survival after surgery. Commercially available zebrafish can vary considerably in health and thus survival after SCI.

2.4. Methods

2.4.1. Preparation of zebrafish for surgery

1) Make a plate of crushed ice before the surgery. Make the surface flat. Carve a 0.5 cm × 2 cm plastic groove into the ice. Place a piece of thin filter paper (2 cm × 2 cm) onto the groove so that the position of the fish will be fixed during surgery (Fig. 2A).
2) Quickly place the zebrafish in tricaine solution. After 3−5 min, upon cessation of respiratory movements of the

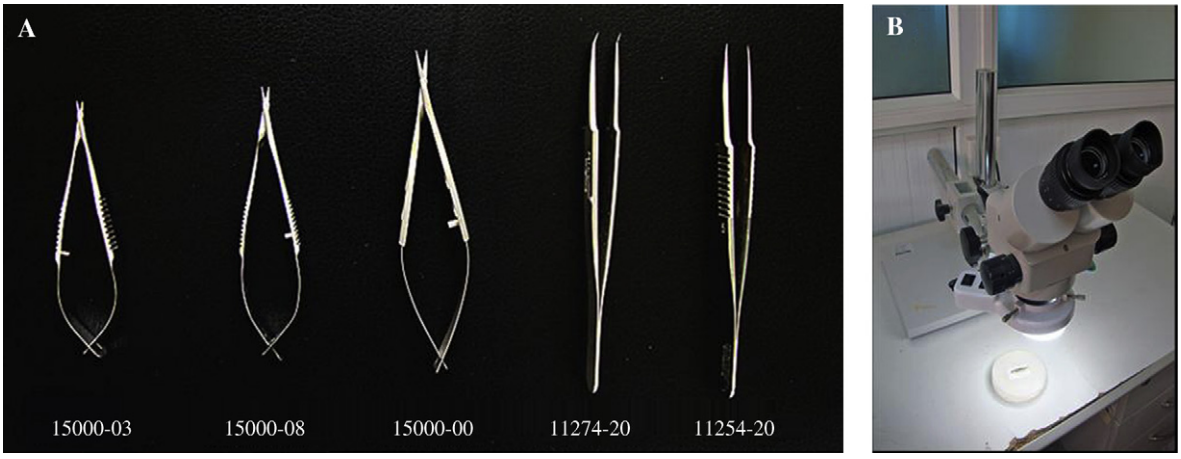

Fig. 1. Equipment and instruments for zebrafish SCI.
A: forceps and spring scissors. **B**: stereomicroscope.

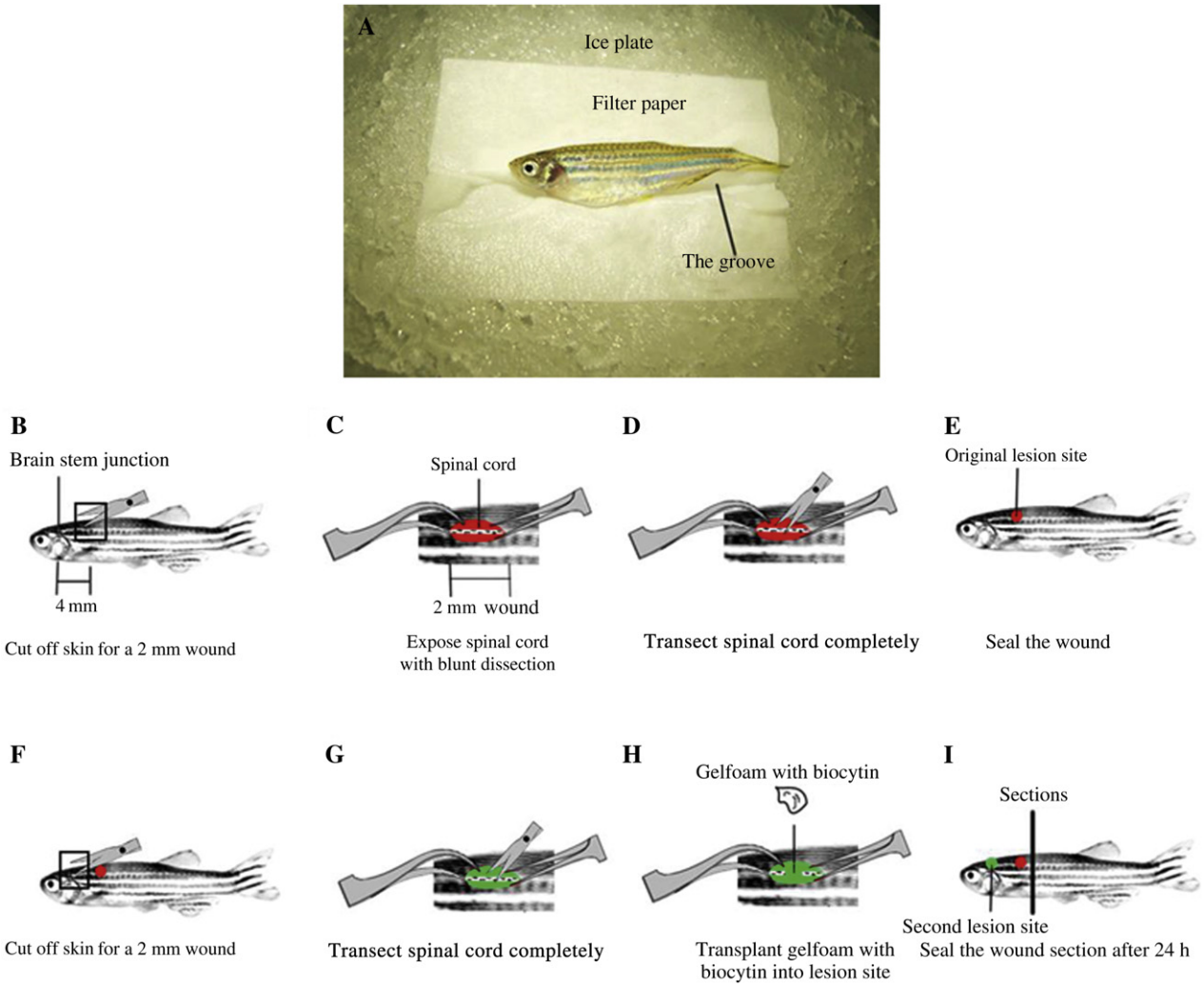

Fig. 2. SCI surgery procedure.
A: preparation before surgery: the fish is placed on an ice plate, head left and body side up. **B**−**E**: experimental steps for SCI in zebrafish. **F**−**I**: experimental steps for the second SCI for axonal anterograde tracing.

opercula, transfer the fish into the filter paper-containing groove on ice, placing the fish on its side with the head pointing left.

!Caution If fish exhibits tremors on ice, prolong the time of anaesthesia until the fish no longer moves. Only then the surgical operation can proceed (Table 1).

2.4.2. SCI and sham surgery (performed under the stereomicroscope)

1) Use a pair of forceps (11254-20) to remove 3−4 squamae at a distance 4 mm caudal to the brainstem/spinal cord transitional junction, then cut off the skin and muscle with the larger spring scissor (15000-08) (Fig. 2B).
2) Expose the spinal cord of the fish using the blunt dissection microforceps (11274-20, 11254-20) (Fig. 2C).

!Caution Handling must be very gentle to avoid inadvertent injuries to the spinal cord, vertebrae or blood vessels. The older the fish are, the better they take the surgery, but on the other hand they do not regenerate as readily as young adult fish.

3) Insert the minor spring scissor (15000-03) into the wound carefully and completely transect the spinal cord (Fig. 2D).

!Caution The transection must be done in a single cut. Repeated cutting will prevent the zebrafish from recovering. The operator must be very careful, during the surgery, to avoid damaging the bone structures under the spinal cord, which encompass the ventral vertebrae (centrum). The blade of the minor spring scissors should avoid hitting these bones during the transection procedure. If the bone vertebrae are damaged, the chances for recovery after surgery are reduced and/or lost.

4) Quickly seal the wound with Histoacryl, and place the zebrafish back into the water. All injured fish need to be kept individually (Fig. 2E).

!Caution Histoacryl quickly solidifies upon exposure to air. Keep the bottleneck of the tube clean for the next use.

Table 1
Trouble shooting

Step	Problem	Possible reason	Solution
2.4.2 2), 3)	Bleeding in wound	Surgical equipment has injured the vessels	Dissect muscles more carefully
2.4.2 4)−2.4.3 2), 2.4.5 4)	Bleeding in fish scales and high death rate	Zebrafish are not healthy Operation skill is not good enough	Buy a new batch from the supplier Practice operation skill
2.4.2 2)	Zebrafish has a "C" shape or "S" shape	The operation may have seriously hurt ventral vertebra or vessels	Zebrafish cannot be used in experiments
2.4.5 8)	No positive signals	Sections are from tissue located more than 4 mm caudal to original lesion site	Prepare sections within 4 mm caudal to original lesion site

5) The sham surgery is performed identically to the SCI surgery except that in the 2.4.2 3) step, the spinal cord is only exposed but not transected.

!Caution Three days after surgery, the sham-injured fish can be traced for swimming behavior, since the distance swum by the sham-injured fish does not differ from the same fish before surgery, as opposed to the fish with transection.

2.4.3. Maintenance of fish after SCI

1) Injured fish are kept individually in 28°C water with anti-fungal agents for 3 days after surgery. The water needs to be changed completely daily until the fish are euthanized.

!Caution All surgical instruments should be cleaned with 75% ethanol after the surgery.

2) Three days after surgery, the fish are fed. Feeding before this time is not recommended since the fish do not respond to food. In addition, providing food that is not eaten may increase fungal pollution, which will elevate the mortality rate. It is advised to powder the food and blow it to the zebrafish through a small pipette in front of the fish.

2.4.4. Analysis of free swimming behavior

Swimming abilities of spinal cord-injured zebrafish and sham-injured zebrafish are evaluated weekly after SCI for 6 consecutive weeks. Since fish can vary slightly in their swim speed, it is advisable to measure each fish before the surgery.

1) In each trial, zebrafish are individually placed with a soft net into a brightly illuminated (100 lux) tank (42 cm × 30 cm × 30 cm) filled with aquarium water (5 cm deep) at 25°C. A video camera records the trials from above the tank.
2) Swim paths are tracked with Ethovision software (Noldus, Wageningen, The Netherlands). Total distances of the swim paths in 10-min trials are recorded. Distances swum during 5−10 min are measured for mean distances. Experiments are performed at the same time (between 9:00 and 10:00 am) on all test days. It is sufficient to record each zebrafish once.

!Caution The experimental operator should be blind to the treatment of the animals.

2.4.5. Anterograde tracing of axons from the brainstem

The brainstem contains the neuronal cell bodies that regrow severed axons. Biocytin is applied at the site indicated in Fig. 2 in sham- and SCI-injured zebrafish directly after surgery and at 3, 7, 11, 15, 18, 21, 30 and 42 days after surgery to monitor axonal regrowth. Biocytin is applied to the fish between 9:00 and 10:00 am and at least 3 fish are taken for each time point.

1) Soak a piece of gelfoam (approximately 2 mm × 2 mm × 2 mm) in the biocytin solution and separate it into five equal pieces.
2) Make an incision immediately below the superior border of branchia, which is the approximate site of the brain-stem/spinal cord junction (for the position, see Fig. 2F). The following manipulations are the same as steps 2) and 3) of 2.4.2 performed for SCI (Fig. 2G).
3) Place one piece of the gelfoam soaked in biocytin into the new lesion site (Fig. 2H) and seal the wound with Histo-acryl (Fig. 2I). Then place the fish back into the water.
4) After 24 h, collect the spinal cord tissue 0−4 mm caudal to original lesion site and place the tissue for fixation into freshly prepared 4% formaldehyde in PBS at 4°C overnight.
5) Immerse tissue sample into 15% sucrose for 6 h at 4°C and then embed in O.C.T.

!Caution Bubbles need to be avoided during the embedding.

6) Cut, in a cryostat, the spinal cord coronally into 25 μm thick sections or longitudinally into 16 μm thick sections and place several sections on a glass slide. Dry at 55°C for 2 h.
7) After biocytin labeling, wash the sections in PBS 3 times for 5 min each at room temperature. Biocytin is detected by incubation with Streptavidin-Cy3 (1:200 in PBS) for 2 h at 37°C. The sections are then rinsed in PBS 3 times for 5 min each.
8) A fluorescence or laser confocal microscope is used to microscopic examination.

!Caution At least 3−4 fish in each group are recommended for the biocytin labeling, while at least 8−10 fish in each group are recommended for analysis of swimming behavior.

3. RESULTS

Successful transection will yield the following results.

3.1. Fish posture

The sham and SCI fish will maintain a straight body posture at all times after surgery. Fish with a "C" or "S" shape are most likely the result of major damage (probably

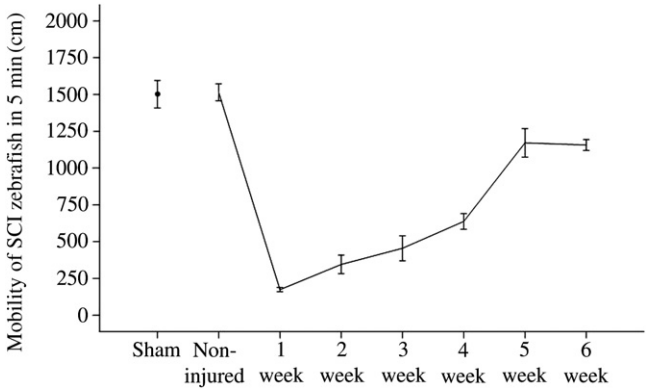

Fig. 3. Weekly mean distances for a 5-min swim path following SCI.
Transection of the spinal cord induced complete loss of swimming ability. However, from the first week after injury, the fish started to regain their ability to swim. Such restoration gradually increased week by week. By the fifth week after injury, the fish reached the peak of recovery in swimming ability when compared to the same fish before SCI. Data represent the mean ± SEM, $n = 9$ for each group.

from injury to the bones) and should not be used for further experiments.

3.2. Swimming ability

In the swim test, we found no difference in 5-min swim distance between sham-injured zebrafish (1501 ± 94 cm) and non-injured zebrafish (1514 ± 58 cm) suggesting that the sham injury did not influence swimming ability. Sham-injured fish were able to swim freely within 3 days after surgery. However, fish after SCI should not be able to swim freely for one week if the transection was complete. In the first week, fish start to regain their ability to swim, and such restoration progresses gradually week by week. The swim distance (174 ± 14 cm) for the first week fish was on average only 11.6% of that of sham-injured zebrafish. Swim distance at the second, third, and fourth week was 345 ± 63 cm, 455 ± 85 cm, 637 ± 53 cm, respectively. The swim distance of the fifth week fish (1171 ± 96 cm) should be equal, on

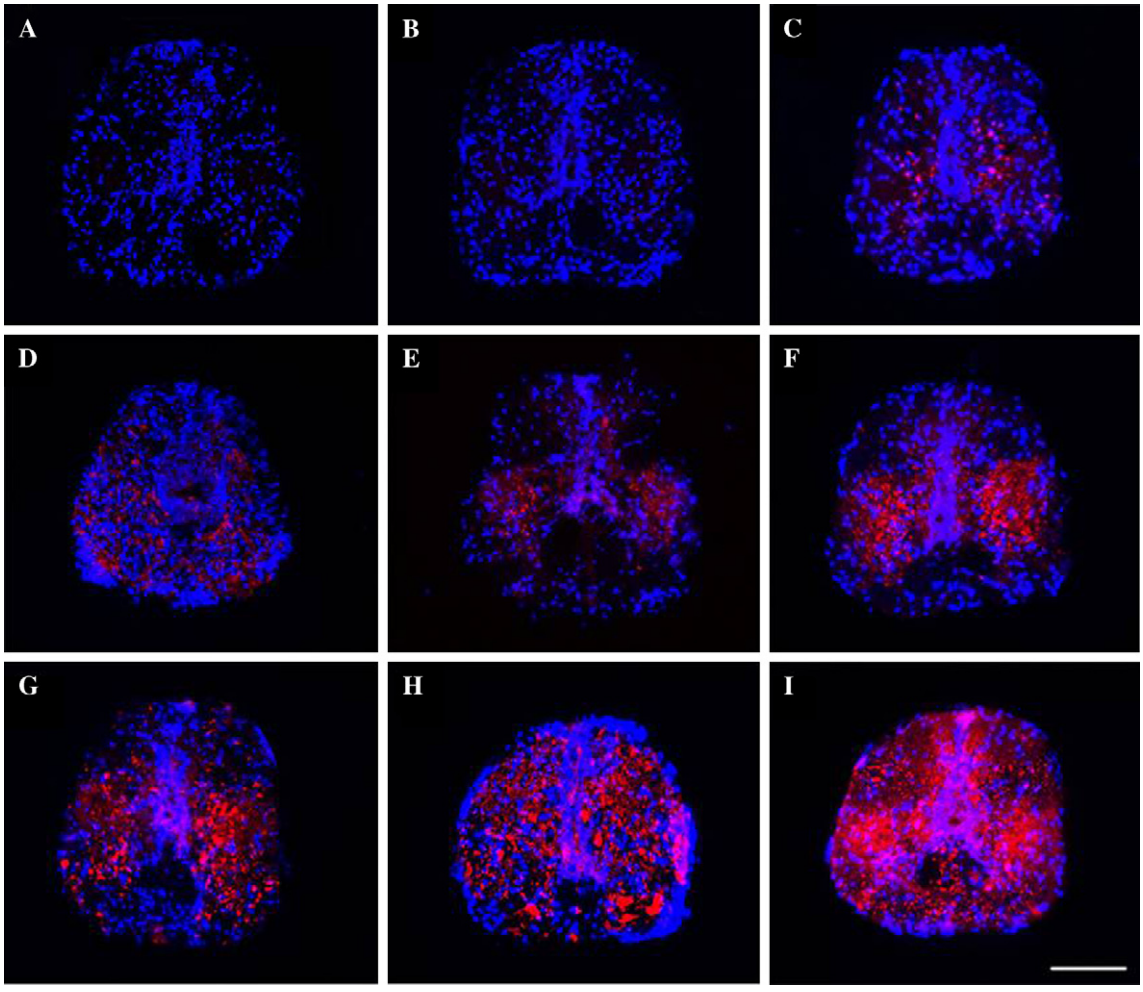

Fig. 4. Axonal anterograde tracing shows the axonal regrowth after SCI in coronal views.
No biocytin labeling was detectable in the spinal cord on day 0 (**A**) or day 7 (**B**) after SCI. Minor axon biocytin labeling appeared in the spinal cord on day 11 (**C**) after SCI. Positive biocytin signals in the spinal cord increased from day 15 (**D**), day 18 (**E**), day 21 (**F**), and day 30 (**G**) following SCI. By day 42 (**H**), biocytin labeling occurred at a similar distance, but with less density compared with the sham control (**I**). Red: biocytin-labeled axons. Blue: DAPI for nuclear staining. Images were taken with a Zeiss microscope (Axio Imager Z1, Zeiss, Oberkochen, Germany). Scale bar is 100 μm.

average, to about 78% of the sham-injured zebrafish (Fig. 3 and Videos S1−S6).

3.3. Anterograde tracing of regrown axons

Axonal regrowth across the lesion site becomes detectable with time after SCI in coronal (Fig. 4) and longitudinal (Fig. 5) views. Samples from day 0 post SCI serve as a negative control, while samples from sham-injured fish serve as a positive control. There are no traced axons in the 7 days post injury group, indicating that axons had not regrown beyond the lesion site by one week after SCI. By 11 days post injury, occasional signals (red) are apparent in both coronal and longitudinal sections. Then, biocytin-labeled axons continually extend over larger distances at 15, 18, 21, and 30 days post injury (Figs. 4 and 5). By 42 days, lengths of regenerated axons in the SCI group are similar, but less in density compared with the sham-injured group (Figs. 4 and 5).

4. DISCUSSION

The survival rate of zebrafish following SCI is strongly influenced by their health status. Zebrafish that have recently been moved to a new environment are prone to branchial bleeding and death following SCI. A water temperature below 15°C also significantly increases the mortality. The health status of zebrafish is difficult to be determined when they are purchased from a supplier. If the mortality reaches over 50%, we recommend changing to a new batch of fish. After SCI, anti-fungal agents will partly improve the health of injured fish which were healthy before SCI, but is of little help with fish that were unhealthy before SCI. Under optimal conditions, fish recover well in swimming ability within 6 weeks after SCI. If fish swim well already within one week after surgery, spinal cord transection was not complete and such fish should not be further evaluated. It should be noted that the mortality of zebrafish after SCI is due largely to the lack of practice. Initially, a mortality rate of 30%−40% has to be taken into account, while with practice, the rate should decrease to 20%.

To monitor successful recovery of swim behavior at the cellular level, there are two ways of tracing: anterograde tracing and retrograde tracing. In anterograde tracing, biocytin is applied *via* gelfoam at the brainstem/spinal cord junction of fish at defined times after SCI. The gelfoam will not be absorbed, but the biocytin will be anterogradely transported to the cell body. Axons descending from brain nuclei, and

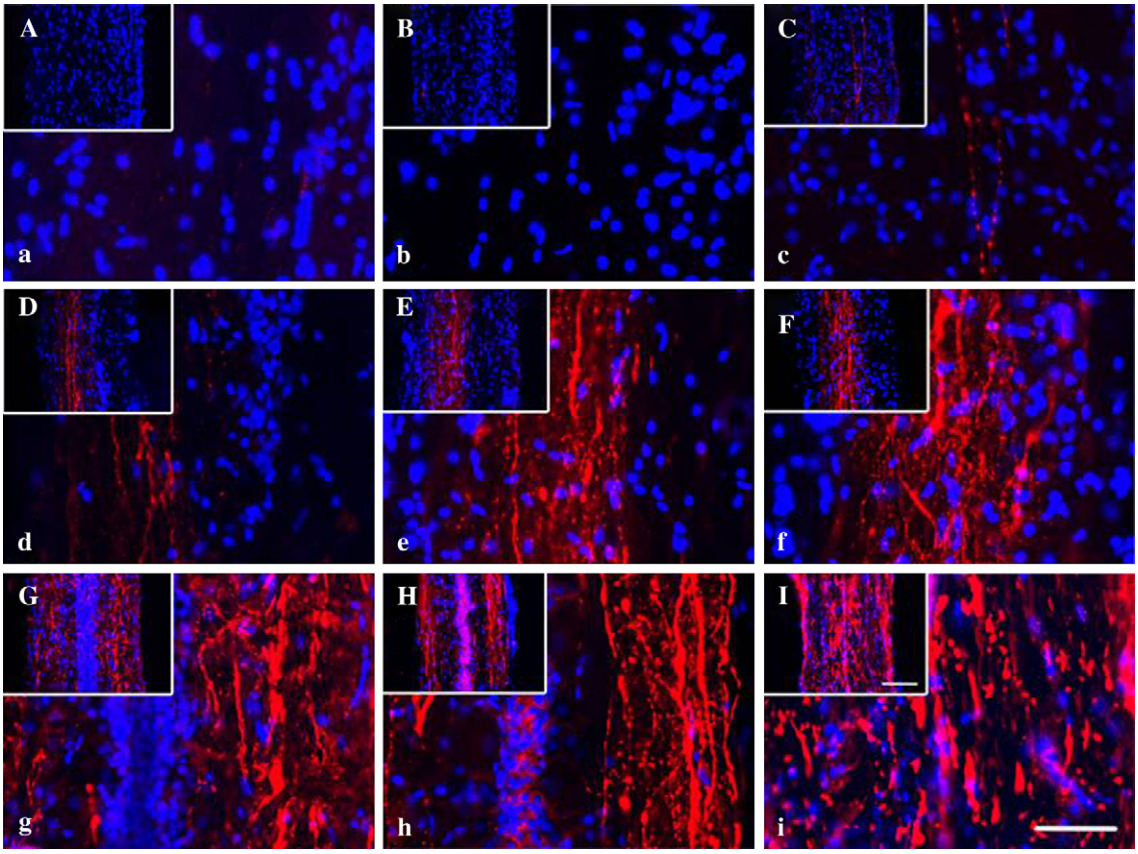

Fig. 5. Axonal anterograde tracing shows axonal regrowth after SCI in longitudinal views.
There are no biocytin-labeled axons in the spinal cord on day 0 (**A**, **a**) or day 7 (**B**, **b**) after SCI. Slight biocytin labeling of axons began to appear in the spinal cord on day 11 (**C**, **c**) after SCI. Positive biocytin signals in the spinal cord progressively increased from day 15 (**D**, **d**), day 18 (**E**, **e**) to day 21 (**F**, **f**) after SCI. On days 30 (**G**, **g**) and 42 (**H**, **h**), biocytin-labeled axons became increasingly thicker compared with day 21 (**F**, **f**). A lower density of axons at day 42 (**H**, **h**) was observed when compared with the sham group (**I**, **i**). Red: biocytin-labeled axons. Blue: DAPI for nuclear staining. Images were taken with a Zeiss microscope (Axio Imager Z1, Zeiss, Oberkochen, Germany). The scale bar is 100 μm in **A**−**I**, and is 50 μm in **a**−**i** (higher magnification of **A**−**I**, respectively).

ascending from locations caudal to transection site to the brainstem junction, will be labeled by the tracer. Sections caudal to the original lesion site can be used to identify these axons (Becker and Becker, 2001; Xue et al., 2004). The mechanism of retrograde axonal tracing is similar to that of anterograde axonal tracing. Biocytin-soaked gelfoam is applied caudal to the original lesion site. Neurons in the different brain nuclei that project to the spinal cord lesion site can then be retrogradely labeled, enabling neural profiles to be detected in sections of the brain (Becker et al., 1997; Xue et al., 2004; Reimer et al., 2009). Here, we only describe anterograde tracing of regrown axons to evaluate successful recovery of zebrafish SCI.

To date, in our system, we have not found a difference in success and survival rates between male and female zebrafish SCI. So there is no gender preference in this model. Whether zebrafish can restore their locomotor ability after repetitive spinal cord injury has yet to be studied.

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SUPPLEMENTARY DATA

Video S1. Swimming ability of zebrafish at 0 week (24 h) after SCI.

Video S2. Swimming ability of zebrafish at 1 week after spinal cord injury.

Video S3. Swimming ability of zebrafish at 3 weeks after SCI.

Video S4. Swimming ability of zebrafish at 6 weeks after SCI.

Video S5. Swimming ability of non-injured zebrafish.

Video S6. Swimming ability of sham-injured zebrafish.

Supplementary video related to this article can be found online at http://dx.doi.org/10.1016/j.jgg.2012.07.010.

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