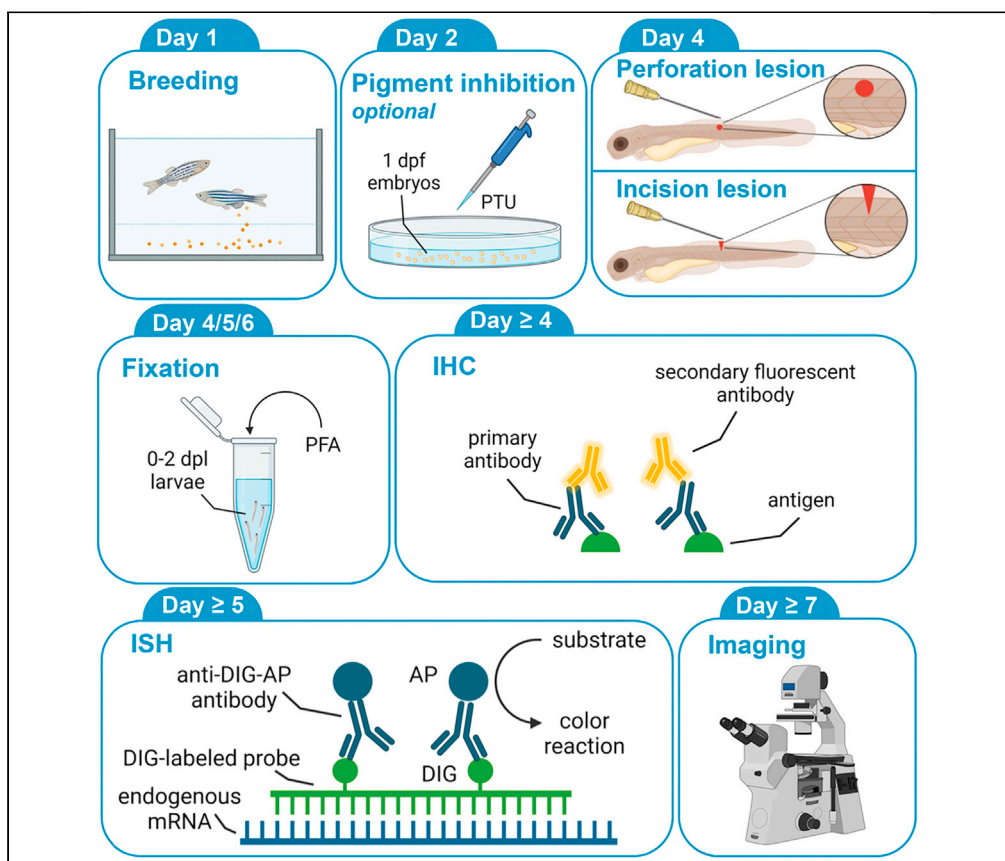


## Protocol

# Mechanical spinal cord transection in larval zebrafish and subsequent whole-mount histological processing



Zebrafish regenerate their spinal cord after injury, both at larval and adult stages. Larval zebrafish have emerged as a powerful model system to study spinal cord injury and regeneration due to their high optical transparency for *in vivo* imaging, amenability to high-throughput analysis, and rapid regeneration time. Here, we describe a protocol for the mechanical transection of the larval zebrafish spinal cord, followed by whole-mount tissue processing for *in situ* hybridization and immunohistochemistry to elucidate principles of regeneration.

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### Highlights

Spinal cord transection in larval zebrafish to study injury and regeneration

Visualization of regeneration-associated gene expression in whole-mounts

Combined immunolabeling of neural cells and ECM proteins in whole-mounts

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## Protocol

## Mechanical spinal cord transection in larval zebrafish and subsequent whole-mount histological processing

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## SUMMARY

Zebrafish regenerate their spinal cord after injury, both at larval and adult stages. Larval zebrafish have emerged as a powerful model system to study spinal cord injury and regeneration due to their high optical transparency for *in vivo* imaging, amenability to high-throughput analysis, and rapid regeneration time. Here, we describe a protocol for the mechanical transection of the larval zebrafish spinal cord, followed by whole-mount tissue processing for *in situ* hybridization and immunohistochemistry to elucidate principles of regeneration. For complete details on the use and execution of this protocol, please refer to Wehner et al. (2017) and Tsata et al. (2021).

## BEFORE YOU BEGIN

The capacity for axon regeneration and functional recovery after spinal cord injury is very limited in mammals. In contrast, zebrafish are able to robustly regrow severed axonal connections and recover swimming function, both at larval and adult stages (Tsata and Wehner, 2021). Thus, zebrafish offer a valuable animal model for studying spinal cord injury and regeneration. Here, we describe procedures to induce mechanical lesions to the larval zebrafish spinal cord, followed by whole-mount immunohistochemistry and *in situ* hybridization for the spatial observation of proteins and gene expression during regeneration. We present two different methodologies to transect the spinal cord of zebrafish larvae: incision and perforation lesion (previously also referred to as stab lesion (Wehner et al., 2017; Tsarouchas et al., 2018; Tsata et al., 2021)). Both methods elicit comparable kinetics of anatomical and functional regeneration and allow for reproducibility, high throughput, and low mortality (Ohnmacht et al., 2016; Wehner et al., 2017, 2018; Tsarouchas et al., 2018; Tsata et al., 2021). After spinal cord transection in 3 days post-fertilization (dpf) zebrafish, axonal regrowth and functional recovery can be observed within two days (Ohnmacht et al., 2016; Wehner et al., 2017). The described tissue processing methods enable the visualization of anatomical regeneration, interactions of regrowing neurites with the lesion environment, as well as lesion-induced alterations in gene expression and protein abundance and localization. Hence, the presented methods provide techniques to elucidate principles of successful spinal cord regeneration in zebrafish, which offer great potential to uncover strategies to foster functional recovery after spinal cord injury in non-regenerating vertebrates.

**Note:** This protocol involves the use of larval zebrafish aged up to 5 dpf. Procedures involving zebrafish older than 5 dpf require approval by an animal ethics committee.



### Zebrafish mating and embryo handling

⌚ Timing: 4 days

1. Breed the desired zebrafish lines to obtain embryos (Westerfield, 2000; Harris et al., 2021) and raise embryos in E3 medium (Brand et al., 2002) at 28.5°C until 3 dpf.

**Note:** A video protocol for mating zebrafish can be found on JoVE (JoVE Science Education Database, 2021). <https://www.jove.com/de/v/5150/zebrafish-breeding-and-embryo-handling>.

**Note:** For training surgical competencies, it is recommended to use transgenic reporter lines that fluorescently label the spinal cord, such as Tg(*elavl3*:*rasmKate2*) (Tsata et al., 2021). This enables visual verification of complete transection using a fluorescence stereo microscope.

**Note:** This protocol involves the use of  $\leq 5$  dpf zebrafish larvae. At these early life-stages the sex cannot be assessed as separate sexes can be detected at its earliest after 21–23 dpf.

**Optional:** Suppress pigmentation by adding 1 mL 50× N-Phenylthiourea (PTU) stock solution to 49 mL E3 medium beginning at 24 h post-fertilization (hpf). Maintain zebrafish in E3 medium containing PTU throughout the experiment.

**Note:** Mechanical spinal cord lesions in the larval zebrafish induce hyperpigmentation of the wound, which may impair imaging of *in situ* hybridization and immunohistochemistry signals in whole-mount tissues. It is therefore recommended to suppress pigmentation during development and regeneration. This can be achieved by using pigmentation mutant strains (e.g., *crystal* (Antinucci and Hindges, 2016)) or by treating zebrafish with pigmentation-suppressing chemical compounds, such as PTU, which inhibits melanogenesis. To avoid potential adverse effects on development, PTU should not be added to the E3 medium before 24 hpf. Note, however, that pigmentation suppression is not recommended when lesioned animals will be assessed for recovery of swimming function using a video tracking platform (Wehner et al., 2017; Tsata et al., 2021).

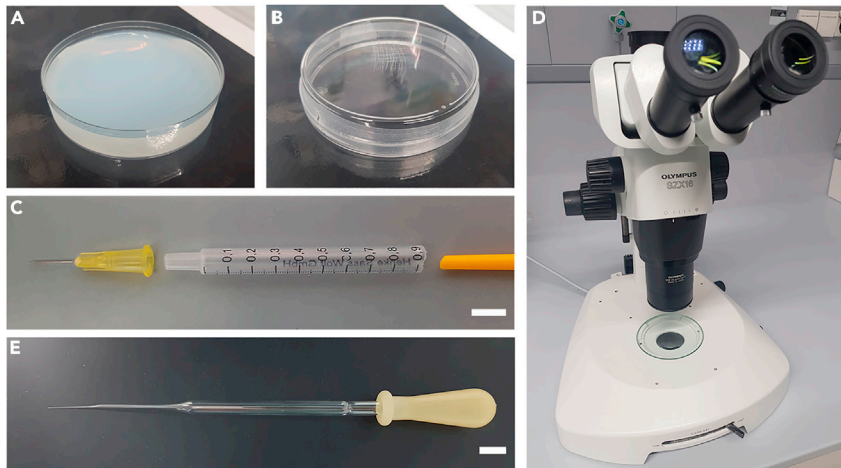
### Preparation of surgery plates, micro scalpels and aspiration pipettes for spinal cord lesions

⌚ Timing: 30 min

To perform spinal cord lesions in zebrafish larvae, two types of surgery plates are required; one for perforation lesions and one for incision lesions. In addition, micro scalpels and aspiration pipettes also need to be prepared.

2. To prepare the surgery plate for perforation lesions, add 1.5 g agarose powder to 100 mL E3 medium (1.5% agarose w/v) and heat in a microwave until the solution is clear and agarose is completely dissolved. Pour  $\sim 75$  mL of agarose into a 10 cm plastic petri dish and let it harden (Figure 1A). The surgery plate can be reused when stored at 4°C. Cover agarose with E3 medium to avoid desiccation.

**⚠ CRITICAL:** Agarose can become superheated and boil suddenly when removed from the microwave, resulting in burn injuries. Consider using a glass flask that is two to three times the volume of the solution when preparing the agarose to leave room for boiling and expansion. Stop the microwave when the agarose starts to boil and gently swirl the flask until agarose is completely dissolved. Wear a lab coat, safety glasses, and heat-resistant gloves when handling the hot flask containing the agarose.



**Figure 1. Essential equipment for transecting the larval zebrafish spinal cord**

- (A) Surgery plate for perforation lesion: 10 cm petri dish coated with 1.5% agarose in E3 medium.  
 (B) Surgery plate for incision lesion: 10 cm petri dish sealed with parafilm. Note the roughened surface (compact grid) for enhanced wettability.  
 (C) Micro scalpel: 30 G  $\times$   $\frac{1}{2}$ " hypodermic needle, 1 mL syringe with clipped barrel flange, handle of cell scraper (~20 cm long; full length not shown) to extend the syringe length. Scale bar: 1 cm.  
 (D) Stereo microscope for examination of needles and monitoring of larvae during and after surgery.  
 (E) Finely drawn glass pipette with fitted rubber bulb for aspiration of excess E3 medium prior to surgery. Scale bar: 1 cm.

**△ CRITICAL:** The surface of the surgery plate must be sufficiently soft to permit penetration of the needle into the plate when lesioning the spinal cord.

- To prepare the surgery plate for incision lesions, seal the top and bottom of a 10 cm plastic petri dish by surrounding the contour with parafilm (Figure 1B). Roughen the surface on one side of the petri dish by scratching a compact grid pattern using a fine hypodermic needle (Figure 1B). The surgery plate can be reused.

**△ CRITICAL:** Surface roughening enhances the wettability (reduces the contact-angle) of the petri dish, which facilitates aspiration of excess E3 medium. Removal of excess E3 medium results in lateral positioning and immobilization of the zebrafish larva for improved accuracy of the lesion.

- To prepare micro scalpels for spinal cord lesions, attach a 30 G  $\times$   $\frac{1}{2}$ " hypodermic needle to a 1 mL syringe. For better handling of the micro scalpel, clip the syringe's barrel flange and extend the syringe length using the handle of a cell scraper (key resources table) (Figure 1C), which can be plugged into the syringe's barrel after removal of the blade.

**△ CRITICAL:** Prior to surgery, check the micro scalpel under a stereo microscope (Figure 1D) to ensure that the needle tip is not deformed.

**Note:** Using commercially available hypodermic needles allows their rapid replacement when deformed or blunt. It also increases reproducibility of the lesion size as the dimension of the needle tip (tip angle, thickness, width) exhibits limited inter- and intra-batch variability.

- For the aspiration of excess E3 medium prior to surgery, prepare a finely drawn glass pipette. Heat the thin part (close to the shoulder) of a glass Pasteur pipette (230 mm long) in a Bunsen flame. As the glass begins to melt, withdraw the pipette from the flame and manually pull the ends of the pipette quickly and smoothly apart. The resulting drawn pipette should be as straight

**Table 1. Pipetting scheme for *in vitro* synthesis reaction of labelled RNA probes**

Reagent	Final concentration	Amount
Purified cDNA template	≤ 50 ng/μL	≤ 1 μg
NTP digoxigenin-labeling mix, 10×	1×	2 μL
RNA Polymerase Transcription buffer (SP6, T3, or T7), 10×	1×	2 μL
RNA polymerase (SP6, T3, or T7)	~ 2 U/μL	2 μL
RNasin (RNase inhibitor)	~ 2 U/μL	1 μL
Molecular grade H <sub>2</sub> O	n/a	Add to 20 μL
<b>Total</b>		<b>20 μL</b>

as possible. Break the drawn glass pipette at its thinnest part using forceps and fit a rubber bulb on the top of the glass pipette (Figure 1E). Aspiration pipettes can be reused.

△ **CRITICAL:** A narrow pipette opening reduces the suction velocity and prevents larvae from being aspirated. The outer diameter of the pipette opening should be in the range of 200–400 μm.

### Synthesis and purification of labeled RNA probes for *in situ* hybridization

⌚ **Timing:** 1 day

*In situ* hybridization (ISH) allows for the spatial detection of gene expression (mRNA). Labeled antisense RNA probes (e.g., digoxigenin- or fluorescein-labeled) hybridize to their complementary mRNA sequences in cells which express the corresponding gene. After washing away excess probe, RNA-hybrids are detected by immunohistochemistry. In order to synthesize the labeled antisense RNA probe, the cDNA sequence of a gene of interest is required as a template. To enable such *in vitro* synthesis, the linear cDNA templates must contain a promoter for either the SP6, T3, or T7 RNA polymerase at the 3' end. The isolation and preparation of cDNA templates has been described in detail elsewhere (Thisse and Thisse, 2008; Chitramuthu and Bennett, 2013).

- Assemble the reaction mix (work RNase-free) for the *in vitro* synthesis of labeled antisense RNA probe according to the pipetting scheme shown in Table 1.

**Note:** In this protocol, the use of digoxigenin-labeled antisense RNA probes is described. However, fluorescein-labeled antisense RNA probes have also been successfully employed. The incubation times, concentrations, volumes, etc. detailed in this protocol are specific for the reagents listed in the [key resources table](#).

- Incubate the transcription reaction mix for 2 h at 37°C.

**Optional:** Add 1 μL DNase I (1 U/μL), mix and incubate for 30 min at 37°C to digest the cDNA template.

- Stop the reaction by adding 2 μL 0.2 M EDTA (pH 8.0), mix and place on ice.

- Purify the synthesized antisense RNA probe by precipitation:
  - Add 2.5 μL 4 M LiCl and 75 μL ice-cold 100% EtOH to the reaction. Mix thoroughly.
  - Precipitate the reaction for 30 min at –80°C (alternatively, incubate for 2 h at –20°C).
  - Centrifuge at ≥ 16,000 g for 15 min at 4°C to pellet the RNA probe.

**Note:** A white pellet must be clearly visible at the bottom of the tube for successful purification.

- d. Aspirate the supernatant and wash the RNA pellet in 200  $\mu$ L 70% EtOH. Do not vortex.
- e. Centrifuge at  $\geq 16,000$  g for 10 min at 4°C.
- f. Aspirate the supernatant and air-dry the pellet under a fume hood for 2–5 min.

**Note:** Avoid over drying of the RNA pellet as this may decrease its solubility.

- g. Resuspend the RNA pellet in 25  $\mu$ L molecular grade H<sub>2</sub>O and place on ice.
- h. Estimate probe yield and the check integrity of the synthesized antisense RNA probe by running 2  $\mu$ L on a 1% agarose gel. Alternatively, RNA purity and concentration can be assessed using a spectrophotometer.

**Note:** The cDNA template must be digested if RNA purity and concentration are assessed using a spectrophotometer (step 6).

**Note:** Use a specific RNA gel loading dye and let the gel run only briefly. Due to different conformations (single-stranded, secondary structures by intra- and intermolecular pairing), purified RNA may run as one or two bands during gel electrophoresis under nondenaturing conditions.

- i. Add 100  $\mu$ L hybridization buffer to the purified RNA probe, mix and store at  $-20^{\circ}\text{C}$  until further use. This stock solution usually contains  $\sim 150$  ng/ $\mu$ L of purified RNA probe.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Goat anti-rabbit Alexa Fluor 488 (working dilution 1:300)	Thermo-Fisher Scientific	Cat#A32731; RRID: AB_2633280
Goat anti-mouse Alexa Fluor 555 (working dilution 1:300)	Thermo Fisher Scientific	Cat#A11008; RRID: AB_143165
Mouse anti-Tubulin, acetylated (working dilution 1:300)	Sigma-Aldrich	Cat#T6793; RRID: AB477585
Rabbit anti-Collagen XII (working dilution 1:250)	<a href="#">Bader et al. (2009)</a>	n/a
Rabbit anti-Fibronectin (working dilution 1:300)	Sigma-Aldrich	Cat#F3648; RRID: AB_476976
Rabbit anti-Glial Fibrillary Acidic Protein (working dilution 1:300)	Agilent	Cat#Z0334; RRID: AB_10013382
Sheep anti-digoxigenin-AP, Fab fragments (working dilution 1:3000)	Roche	Cat#11093274910; RRID: AB_2734716
Sheep anti-fluorescein-AP, Fab fragments (working dilution 1:3000)	Roche	Cat# 11426338910; RRID: AB_2734723
<b>Chemicals, peptides, and recombinant proteins</b>		
Acetone	VWR	Cat#20066.296
Agarose	Serva Electrophoresis	Cat#11406
BM-Purple	Roche	Cat#11442074001
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A2153
Calcium chloride dihydrate (CaCl <sub>2</sub> · 2 H <sub>2</sub> O)	Sigma-Aldrich	Cat#C8106
Citric acid monohydrate ((CH <sub>2</sub> COOH) <sub>2</sub> · H <sub>2</sub> O)	Sigma-Aldrich	Cat#C1909
Disodium phosphate dihydrate (Na <sub>2</sub> HPO <sub>4</sub> · 2 H <sub>2</sub> O)	Sigma-Aldrich	Cat#71643
DNase I (RNase free, 1 U/ $\mu$ L)	Promega	Cat#M6101
Ethanol (EtOH)	Thermo Fisher Scientific	Cat#10342652
Ethylenediaminetetraacetic acid (EDTA)	PluriSelect	Cat#60-00030-10
Ethyl 3-aminobenzoate methanesulfonate (MS-222)	PharmaQ	Cat#Tricaine PharmaQ
Formaldehyde Solution (16%), Methanol-free	Thermo Fisher Scientific	Cat#28908
Formamide, deionized (HCONH <sub>2</sub> )	Thermo Fisher Scientific	Cat#AM9342

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Glycerol	Sigma-Aldrich	Cat#G5516
Heparin sodium salt	Sigma-Aldrich	Cat#H4784
Lithium chloride (LiCl)	Sigma-Aldrich	Cat#L9650
Magnesium chloride (MgCl <sub>2</sub> )	Sigma-Aldrich	Cat#M8266
Magnesium sulfate heptahydrate (MgSO <sub>4</sub> · 7 H <sub>2</sub> O)	Sigma-Aldrich	Cat#M5921
Methanol (MeOH)	VWR	Cat#1.06009.1000
Methylene Blue solution, 0.05%	Sigma-Aldrich	Cat#319112
Molecular grade H <sub>2</sub> O	Carl Roth	Cat#T143
Monopotassium phosphate (KH <sub>2</sub> PO <sub>4</sub> )	Sigma-Aldrich	Cat#P5655
NBT/BCIP Tablets	Roche	Cat#11697471001
N-Phenylthiourea (PTU)	Sigma-Aldrich	Cat#P7629
NTP digoxigenin-labeling mix (10×)	Roche	Cat#11277073910
Phosphate buffered saline (PBS, 10×)	Sigma-Aldrich	Cat#P5493
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P9333
Proteinase K solution	Invitrogen	Cat#25530049
RNA Gel Loading Dye (2×)	Thermo Fisher Scientific	Cat#R0641
RNA polymerase (SP6)	Roche	Cat#11487671001
RNA polymerase (SP6) transcription buffer (10×)	Roche	Cat#11487671001
RNA polymerase (T3)	Roche	Cat#11031163001
RNA polymerase (T3) transcription buffer (10×)	Roche	Cat#11031163001
RNA polymerase (T7)	Roche	Cat#10881767001
RNA polymerase (T7) transcription buffer (10×)	Roche	Cat#10881767001
RNasin (RNase inhibitor)	Promega	Cat#N251B
Sheep serum	Sigma-Aldrich	Cat#S2263
Sodium azide (NaN <sub>3</sub> )	Sigma-Aldrich	Cat#71289
Sodium chloride (NaCl)	Sigma-Aldrich	Cat#S7653
Sodium citrate tribasic dihydrate ((CH <sub>2</sub> COONa) <sub>2</sub> · 2 H <sub>2</sub> O)	Sigma-Aldrich	Cat#71405
Sodium hydroxide solution 1 M (NaOH)	Sigma-Aldrich	Cat#109137
Transcription buffer (10×)	Roche	Cat#11465384001
Triton™ X-100	Carl Roth	Cat#3051
Trizma hydrochloride (Tris-HCl, NH <sub>2</sub> C(CH <sub>2</sub> OH) <sub>3</sub> · HCl)	Sigma-Aldrich	Cat#T5941
Tween™ 20	Sigma-Aldrich	Cat#P1379
Type V tRNA from wheat germ	Sigma-Aldrich	Cat#R7876
Type VI Torula yeast RNA	Sigma-Aldrich	Cat#R6625
<b>Experimental models: Organisms/strains</b>		
Zebrafish: Tg( <i>elavl3:rasmKate2</i> ) <sup>mps1</sup>	<a href="#">Tsata et al. (2021)</a>	ZFIN: ZDB-ALT-200812-2
Zebrafish: Tg(BAC( <i>pdgfrb:Gal4ff</i> )) <sup>ncv24</sup>	<a href="#">Ando et al. (2016)</a>	ZFIN: ZDB-ALT-160609-3
Zebrafish: Tg(5xUAS:EGFP) <sup>zfl82</sup>	<a href="#">Asakawa et al. (2008)</a>	ZFIN: ZDB-ALT-080528-1
Zebrafish: Tg( <i>her4.3:EGFP</i> ) <sup>y83</sup> (formerly known as Tg( <i>her4.1:EGFP</i> ))	<a href="#">Yeo et al. (2007)</a>	ZFIN: ZDB-ALT-070612-3
Zebrafish: AB wildtype	EZRC	ZFIN: ZDB-GENO-960809-7
Zebrafish: WIK wildtype	EZRC	ZFIN: ZDB-GENO-010531-2
<b>Other</b>		
27 G × 3/4" hypodermic needle	BD Biosciences	Cat#3086999
30 G × 1/2" hypodermic needle	BD Biosciences	Cat#305106
Bunsen burner (e.g., Fireboy plus)	Integra Bioscience	Cat#144000
Cell scraper ('20 cm stick')	TPP	Cat#99002
Centrifuge (e.g., Heraeus Fresco 21)	Thermo Fisher Scientific	Cat#75002426
Cover slip	VWR	Cat#631-1570
Fluorescence stereo microscope	Leica Camera AG	M205 FCA
Forceps	n/a	n/a
Glass Pasteur pipette	Hecht Assistent	Cat#40567002
Laser scanning confocal microscope	Zeiss	LSM-980
Micro scissors	Fine Science Tools	Cat#15000-08
Microscope slide	VWR	Cat#630-0950

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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Petri dish (35 mm)	Greiner Bio One	Cat#627102
Petri dish (100 mm)	Greiner Bio One	Cat#664102
Reaction tube (1.5 mL)	Carl Roth	Cat#CH76.1
Reaction tube (2 mL)	Carl Roth	Cat#CK06.1
Rubber bulb	Merck	Cat#Z111589
See-saw rocking shaker	neoLab	Cat#7-0952
Spectrophotometer (e.g., NanoDrop One <sup>C</sup> )	Thermo Fisher Scientific	Cat#ND-ONEC-W
Stereo microscope	Olympus	SZX 16 V2.0
ThermoMixer C	Eppendorf	Cat#EP5382000023
Transfer pipette (3 mL)	Alpha Laboratories	Cat#LW4111
Waterbath (e.g., Precision GP 20)	Thermo Fisher Scientific	Cat#TSGP20
Whatman indicator papers	Sigma-Aldrich	Cat#WHA10362000

**MATERIALS AND EQUIPMENT**

**60× E3 medium stock solution**

Reagent	Final concentration	Amount
NaCl	0.3 M	172 g
KCl	10.2 mM	7.6 g
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	19.8 mM	29 g
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	19.8 mM	49 g
ddH <sub>2</sub> O	n/a	Add to 10 L
<b>Total</b>		<b>10 L</b>

**Note:** Mix thoroughly and store at 4°C for up to 6 months.

**1× E3 medium (working solution)**

Reagent	Final concentration	Amount
60× E3 stock solution	1×	166 mL
Methylene Blue solution, 0.05%	0.00002%	4 mL
ddH <sub>2</sub> O	n/a	Add to 10 L
<b>Total</b>		<b>10 L</b>

**Note:** Mix thoroughly, adjust pH to 7.2 with NaOH and store at room temperature (RT; 20°C–22°C) for up to one month.

**50× N-Phenylthiourea (PTU) stock solution**

Reagent	Final concentration	Amount
PTU	50×	150 mg
ddH <sub>2</sub> O	n/a	Add to 100 mL
<b>Total</b>		<b>100 mL</b>

**Note:** Mix thoroughly and store protected from light at 4°C for up to 6 months.

△ **CRITICAL:** Acutely toxic, skin sensitizing. May be fatal if swallowed and may cause an allergic skin reaction. Wear protective gloves during preparation and application.



**Phosphate buffered saline (PBS)**

Reagent	Final concentration	Amount
10x PBS	1x	100 mL
ddH <sub>2</sub> O	n/a	900 mL
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly and store at RT for up to 6 months. The pH of PBS should be 7.4. If necessary, pH can be adjusted using HCl or NaOH.

**1% MS-222 stock solution**

Reagent	Final concentration	Amount
MS-222	1%	10 g
PBS	n/a	Add to 1 L
<b>Total</b>		<b>1 L</b>

**Note:** MS-222 is acidic in aqueous solution and should be buffered with imidazole, sodium hydrogen phosphate or sodium hydroxide (Topic Popovic et al., 2012). Hence, dissolve in appropriate buffer (here PBS), mix thoroughly and adjust pH to 7.2–7.4 with NaOH or HCl. Store at 4°C for up to 6 months.

△ **CRITICAL:** This is a controlled substance that should be obtained through your animal facility veterinarian. Please follow the instructions provided by your institution's ethical committee.

**4% Formaldehyde (PFA)**

Reagent	Final concentration	Amount
Formaldehyde solution 16%, deionized	4%	10 mL
PBS	n/a	30 mL
<b>Total</b>		<b>40 mL</b>

**Note:** Mix thoroughly and store at 4°C for up to one week.

△ **CRITICAL:** Highly toxic substance. Harmful if swallowed, in contact with skin or if inhaled. Causes serious eye and skin irritation. May cause an allergic skin reaction. Suspected of causing genetic defects. May cause cancer and respiratory irritation. Wear protective gloves, protective clothing, eye and face protection.

**Phosphate buffered saline Tween™ 20 (PBST)**

Reagent	Final concentration	Amount
10x PBS	1x	100 mL
Tween™ 20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	899 mL
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly and store at RT for up to 6 months.

**20× Saline sodium citrate stock solution (20× SSC)**

Reagent	Final concentration	Amount
NaCl	3 M	175.32 g
Sodium citrate tribasic dihydrate; (CH <sub>2</sub> COONa) <sub>2</sub> · 2 H <sub>2</sub> O	0.3 M	88.23 g
ddH <sub>2</sub> O	n/a	Add to 1 L
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly, adjust pH to 7.0 with HCl and store at RT for up to 12 months. Discard if precipitates form.

**2× Saline sodium citrate Tween™ 20 (2× SSCT)**

Reagent	Final concentration	Amount
20× SSC	2×	100 mL
Tween™ 20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	899 mL
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly and store at RT for up to 12 months. Discard if precipitates form.

**0.2× Saline sodium citrate Tween™ 20 (0.2× SSCT)**

Reagent	Final concentration	Amount
20× SSC	2×	10 mL
Tween™ 20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	989 mL
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly and store at RT for up to 12 months. Discard if precipitates form.

**Hybridization buffer**

Reagent	Final concentration	Amount
20× SSC	5×	250 mL
Type VI Torula yeast RNA or type V tRNA from wheat germ	500 µg/mL	500 mg
Heparin	50 µg/mL	50 mg
Tween™ 20	0.1%	1 mL
Citric acid monohydrate; (CH <sub>2</sub> COOH) <sub>2</sub> · H <sub>2</sub> O	9 mM	1.89 g
ddH <sub>2</sub> O	n/a	Add to 500 mL
Formamide, deionized	50%	500 mL
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly. The final pH should be 6.0–6.5 and usually no pH adjustment is required. Determine pH using indicator paper. Store at –20°C for up to 12 months.

**△ CRITICAL:** Formamide is toxic and carcinogenic, and may affect fertility or the unborn child if swallowed. Avoid inhalation and contact with the eyes and skin. Wear protective gloves, clothes, eye and face protection. Dispose of contents in an approved waste disposal plant.

#### SSCT/formamide mix

Reagent	Final concentration	Amount
2x SSCT	50%	4 mL
Formamide, deionized	50%	4 mL
<b>Total</b>		<b>8 mL</b>

**Note:** Mix thoroughly. Volumes given above are sufficient for one sample. Always prepare SSCT/formamide mix fresh according to the number of samples.

⚠ **CRITICAL:** Formamide is toxic and carcinogenic. See precautions above.

#### ISH blocking solution

Reagent	Final concentration	Amount
Sheep serum	5%	200 $\mu$ L
BSA	10 mg/mL	40 mg
PBST	n/a	Add to 4 mL
<b>Total</b>		<b>4 mL</b>

**Note:** Mix thoroughly. Sheep serum should be heat inactivated at 60°C for 1 h, aliquoted and stored at –20°C. Prior to use, thaw aliquot and centrifuge at  $\geq 16,000$  g for 5 min to pellet coagulated serum. Use only the supernatant. Volumes given above are sufficient for one sample. Always prepare fresh according to the number of samples.

#### NTMT staining buffer

Reagent	Final concentration	Amount
Tris-HCl	100 mM	31.5 g
MgCl <sub>2</sub>	50 mM	95 mg
NaCl	100 mM	117 mg
Tween™ 20	0.1%	20 $\mu$ L
ddH <sub>2</sub> O	n/a	Add to 20 mL
<b>Total</b>		<b>20 mL</b>

**Note:** Mix thoroughly. Always prepare fresh.

#### Phosphate buffered saline Triton™ X-100 (PBTx)

Reagent	Final concentration	Amount
10x PBS	1x	100 mL
Triton™ X-100	1%	10 mL
ddH <sub>2</sub> O	n/a	890 mL
<b>Total</b>		<b>1 L</b>

**Note:** Mix thoroughly and store at RT for up to 6 months.

#### IHC blocking solution

Reagent	Final concentration	Amount
BSA	4%	160 mg
PBTx	n/a	Add to 4 mL
<b>Total</b>		<b>4 mL</b>

**Note:** Mix thoroughly. Volumes given above are sufficient for one sample. Always prepare fresh according to the number of samples.

### STEP-BY-STEP METHOD DETAILS

#### Transection of the larval zebrafish spinal cord

⌚ Timing: 1 h

This protocol describes two methods for the mechanical transection of the larval zebrafish spinal cord: perforation lesion and incision lesion. Perforation lesions leave the dorsal edge of the larval trunk intact and may be more suitable for downstream histological processing than incision lesions. Incision lesions result in clean lesion edges and less tissue perturbation, which may be beneficial for live imaging. To prevent notochord damage and ensure the complete transection of the spinal cord, surgeries are performed under visual control at high magnification using a stereo microscope (Figure 1D). The relevant anatomical structures of a 3 dpf zebrafish larva are indicated in Figure 2A.

1. Anesthetize zebrafish larvae (3 dpf) in E3 medium containing 0.02% Ethyl 3-aminobenzoate methanesulfonate (MS-222/Tricaine). Larvae are sufficiently anesthetized when no response to touch can be observed.
2. Prepare a recovery dish containing E3 medium without MS-222.

**Note:** To suppress hyperpigmentation of the wound, add PTU to the E3 medium (before you begin step 1).

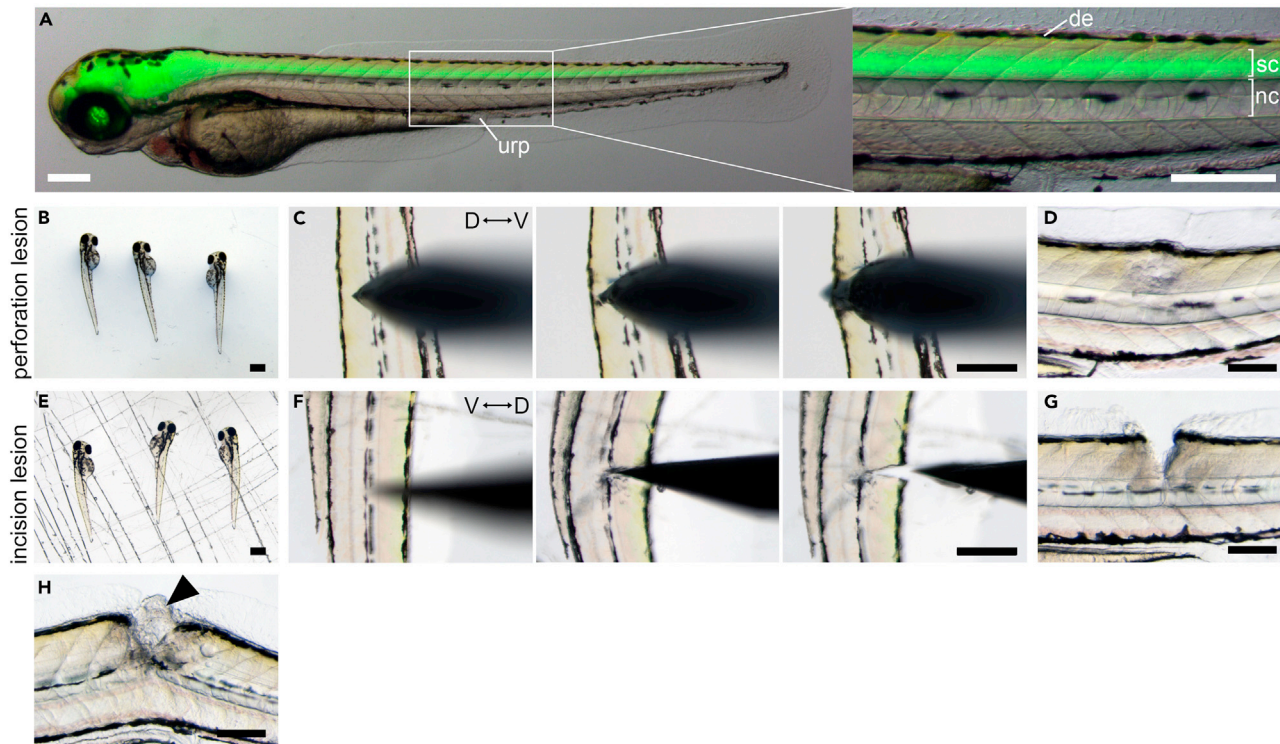
3. Perforation lesion:
  - a. Transfer anesthetized larvae to the surgery plate (agarose-coated petri dish; Figure 1A) using a transfer pipette.
  - b. Remove excess E3 medium using an aspiration pipette (Figure 1E). The trunks of the larvae will settle into a lateral position (Figure 2B).
  - c. Approach the larva with the micro scalpel (Figure 1C) from the ventral side, at the level of the urogenital pore. The needle bevel should be facing up (towards the dorsal side of the larva). Push the tip of the needle through the trunk immediately dorsal to the notochord to transect the spinal cord (Figures 2C and 2D, Methods video S1). Take care not to injure the notochord.

**Note:** Usually, the spinal cord should be transected after a single perforation. However, untrained experimenters may require several repetitions of step 3c. For training purposes, we recommend the use of transgenic reporter lines that fluorescently label the spinal cord (before you begin step 1), which enables visual verification of complete transection using a fluorescence stereo microscope.

- d. Gently flush lesioned larvae off the surgery plate into the recovery dish using a transfer pipette filled with E3 medium.

**Note:** Steps 3a to 3d require approximately 10–20 s per larva.

- e. Keep lesioned larvae in a 28.5°C incubator for recovery.
  - f. Remove sick and dead larvae on the same day and subsequently check lesioned larvae daily.
4. Incision lesion:
    - a. Transfer anesthetized larvae to the surgery plate (roughened surface of a sealed plastic petri dish; Figure 1B) using a transfer pipette.



**Figure 2. Mechanical spinal cord transection in larval zebrafish**

(A) Lateral view of a Tg(*her4.3:EGFP*) transgenic zebrafish larva at 3 dpf, labeling ependymoradial glia cells (astroglia-like cells) in the spinal cord with GFP protein (green). Anatomical structures relevant for the described protocol are indicated. Abbreviations: de, dorsal edge; nc, notochord; sc, spinal cord; urp, urogenital pore.

(B) Larvae placed in lateral position on the surgery plate in preparation for perforation lesion.

(C) Perforation lesion procedure to transect the larval zebrafish spinal cord. Images shown are single frames of the [Methods video S1](#). Lateral view; dorsal is left. Abbreviations: D, dorsal; V, ventral.

(D) Representative image of a perforation lesion immediately after injury. Note that the dorsal edge of the trunk remains intact. Lateral view; rostral is left, dorsal is up.

(E) Larvae placed in lateral position on the surgery plate in preparation for incision lesion.

(F) Incision lesion procedure to transect the larval zebrafish spinal cord. Images shown are single frames of the [Methods video S2](#). Lateral view; dorsal is right. Abbreviations: D, dorsal; V, ventral.

(G) Representative image of an incision lesion immediately after injury. Note that the notochord is left intact. Lateral view; rostral is left, dorsal is up.

(H) Extensive injury to the notochord following an incision lesion leads to the formation of a bulgy tissue structure (arrowhead). Lateral view; rostral is left.

(A–H) Scale bars: 500  $\mu$ m (B, E), 200  $\mu$ m (A, C, F), 100  $\mu$ m (D, G, H)

- b. Remove excess E3 medium using an aspiration pipette ([Figure 1E](#)). The trunks of the larvae will settle into a lateral position ([Figure 2E](#)).
- c. Approach the larva with the micro scalpel ([Figure 1C](#)) from the dorsal side, at the level of the urogenital pore. The needle bevel should be facing sideways. Induce an incision into the dorsal trunk spanning from the notochord to the dorsal edge ([Figures 2F and 2G](#), [Methods video S2](#)). Take care not to injure the notochord.

**△ CRITICAL:** Larvae that undergo excessive damage to the notochord typically show the formation of a bulging tissue structure within hours after surgery, which can be of varying size and is highly inhibitory to regeneration ([Figure 2H](#); arrowhead). Those animals should be excluded from further analysis and terminally anesthetized by adding an overdose of MS-222 to the E3 medium (final concentration of  $\geq 0.1\%$ ).

- d. Gently flush lesioned larvae off the surgery plate into the recovery dish, using a transfer pipette filled with E3 medium.

**Note:** Steps 4a–4d require approximately 10–20 s per larva.

- e. Keep lesioned larvae in a 28.5°C incubator for recovery.
- f. Remove sick and dead larvae on the same day and subsequently check lesioned larvae daily.

△ **CRITICAL:** Avoid desiccation of the larvae as this will result in exacerbated tissue damage and high mortality rates (Problem 2). Ensure that a sufficient volume of E3 medium is left on the surgery plate.

### Whole-mount *in situ* hybridization

⌚ **Timing:** 3–4 days

The following protocol describes *in situ* hybridization (ISH) to detect specific mRNAs in whole-mount zebrafish larvae. After spinal cord lesion (0–2 days post-lesion; dpl), zebrafish larvae are fixed with paraformaldehyde (PFA) to prepare them for labeling. Fixed larvae are permeabilized and incubated with the labeled antisense RNA probe (**before you begin** steps 6 and 7) which hybridize to their complementary mRNA sequence in cells expressing the corresponding gene. RNA hybrids are visualized using immunohistochemistry (Figure 3). The step-by-step details are described in this section. Note that the protocol provides efficient probe penetration in whole-mount 5 dpf zebrafish larvae as previously reported (Wehner et al., 2017).

Unless stated otherwise, perform all incubations at room temperature (RT; 20°C–22°C) without agitation and use a vacuum pump to draw media. Take care not to aspirate larvae. Small volumes can be aspirated using a fine hypodermic needle ( $\leq 27\text{ G} \times \frac{3}{4}\text{''}$ ) attached to the vacuum pump. The volume of the solutions should always be sufficient to cover the larvae. Unless stated otherwise, we recommend using 2 mL solution per tissue condition.

5. Terminally anesthetize larvae by adding an overdose of MS-222 to the E3 medium (final concentration of  $\geq 0.1\%$ ).
6. Transfer larvae to a 2 mL reaction tube using a transfer pipette.

△ **CRITICAL:** Use 2 mL reaction tubes to allow for sufficient washing volumes.

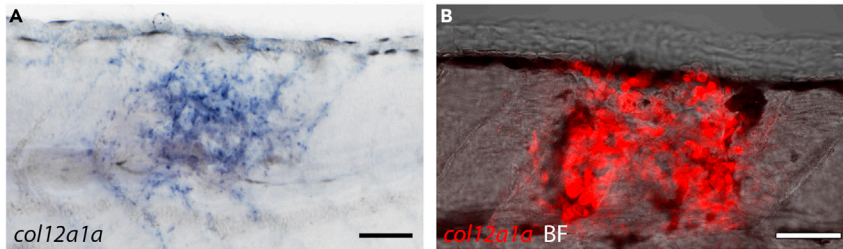
7. Fix larvae in 4% PFA at 4°C for 14–18 h. Place reaction tubes in a tube rack tilted by 90°.
8. Wash larvae 2× in PBST for 1 min each.
9. Dehydrate larvae stepwise by successive incubation in 25% MeOH-PBST, 50% MeOH-PBST, 75% MeOH-PBST, and 100% MeOH for 5 min each.

**Note:** Samples can be stored in 100% MeOH at –20°C for several months.

△ **CRITICAL:** MeOH is acutely toxic and flammable. It damages organs when in direct contact, if swallowed or inhaled. Wear protective gloves, protective clothing, eye, and face protection.

10. Rehydrate samples stepwise by successive incubation in 75% MeOH-PBST, 50% MeOH-PBST, 25% MeOH-PBST, and PBST for 5 min each.
11. Wash larvae 2× in PBST for 1 min each.
12. For permeabilization, incubate larvae in PBST containing 40 µg/mL Proteinase K for 30 min. Place reaction tubes in a tube rack tilted by 90°.

△ **CRITICAL:** Due to batch-to-batch variations of Proteinase K activity, its concentration and incubation time need to be tested and adjusted for each batch to avoid over or under



**Figure 3. Representative examples of whole-mount ISH in spinal cord lesioned larval zebrafish**  
(A and B) ISH reveals local upregulation of *col12a1a* expression in the lesion site at 1 dpl (perforation lesion) as compared to adjacent unlesioned trunk tissue. *col12a1a* mRNA expression was visualized using either NBT/BCIP substrate (A; blue) or Fast Red substrate (B; red). Note that PTU was used to suppress developmental and lesion-induced pigmentation in (A) while the larva in (B) was untreated. Images shown are brightfield recordings (A) or a maximum intensity projection of a confocal image (B) of the lesion site (lateral view; rostral is left, dorsal is up). Abbreviation: BF, brightfield. Scale bars: 50  $\mu$ m.

digestion of the samples. As a starting point, we recommend 40  $\mu$ g/mL for 30 min to permeabilize 3–5 dpf (0–2 dpl) zebrafish.

13. Stop the Proteinase K digestion by washing larvae 2 $\times$  in PBST for  $\leq$  1 min each.
14. Re-fix larvae by incubation in 4% PFA for 15 min.

**Note:** Do not fix longer than 15 min, otherwise hybridization efficiency may be suboptimal.

15. Wash larvae 5 $\times$  in PBST for  $\leq$  1 min each.
16. For prehybridization, incubate larvae in hybridization buffer (preheated to 66°C) for a minimum of 1 h at 66°C in a water bath. This step can be extended up to several hours.
17. In the meantime, prepare hybridization mixes (1 mL hybridization buffer + 5  $\mu$ L RNA probe stock solution for a 1:200 dilution) in 2 mL reaction tubes.

**Note:** Optimal concentration of the RNA probe needs to be determined. As a starting point, we recommend a 1:200 dilution of the stock solution (before you begin step 7i).

18. Preheat the hybridization mix to 66°C for 10 min on a thermomixer under vigorous shaking at  $\sim$ 900 rpm.
19. Aspirate media and add 1 mL of preheated hybridization mix to each reaction tube. Hybridize at 66°C in a water bath for 14–18 h.

For the following washing steps, buffers should always be preheated in a water bath to hybridization temperature (66°C). The reaction tubes with the hybridized larvae should only be briefly taken out of the water bath for aspiration of solutions to avoid significant temperature drops.

20. Washing:
  - a. Aspirate the hybridization mix and wash samples in hybridization buffer for 20 min.

**Note:** When stored at  $-20^{\circ}$ C, RNA probes can be reused several times before the signal of the staining reaction weakens (step 26). We typically reuse RNA probes twice.

- b. Freshly prepare the SSCT/formamide mixture and wash larvae 3 $\times$  at 66°C for 20 min each.
- c. Wash 2 $\times$  in 2 $\times$  SSCT buffer at 66°C for 20 min each.
- d. Wash 4 $\times$  in 0.2 $\times$  SSCT buffer at 66°C for 30 min each.

△ **CRITICAL:** This is the most stringent washing step and washing time should not be extended.

e. Wash 2× in PBST for ≤1 min each.

**Note:** This, and all following steps, are again carried out at RT without agitation unless stated otherwise.

21. Incubate larvae in blocking solution for at least 1 h. Place the tubes on a see-saw rocking shaker with low agitation at ~10 rpm.
22. Dilute anti-digoxigenin-alkaline phosphatase antibody (anti-digoxigenin-AP) 1:3000 in blocking solution.

**Note:** This protocol describes the use of digoxigenin-labeled RNA probes, but can be also applied for fluorescein-labeled RNA probes. If fluorescein-labeled probes are used, apply appropriate antibody (anti-fluorescein-AP) and keep samples in the dark.

23. Incubate larvae in ≥ 1 mL antibody solution at 4°C for 14–18 h. Keep tubes in a 90° tilted tube rack, optionally on a see-saw rocking shaker with low agitation at ~10 rpm.

△ **CRITICAL:** Ensure that the larvae are fully immersed in antibody solution at all times.

24. Wash on a see-saw rocking shaker with low agitation at ~10 rpm:
  - a. 2× in PBST for 1 min each.
  - b. 6× in PBST for 20 min each.

**Note:** Recover the antibody solution; it can be reused several times when stored at 4°C. For storage, add sodium azide at a final concentration of 0.02% (3 mM) to prevent microbial growth.

25. Freshly prepare NTMT staining buffer. Wash larvae in NTMT staining buffer 2× for 5 min each to replace PBST and increase the pH to 9.0–9.5.

**Note:** The alkaline phosphatase (AP) enzyme is most stable between pH 7.5–9.5, with pH 9.0 being optimal.

26. Staining reaction: The anti-digoxigenin antibody is conjugated to AP, which catalyzes chromogenic reactions. Various AP substrates, which yield different colors can be used, such as nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and BM-Purple to obtain blue color, 2-(4-iodophenyl)-5-(4-nitrophenyl)-3-phenyltetrazolium chloride (INT)/BCIP for brown color and Fast Red for a red precipitate which is also fluorescent in the red spectrum.
  - a. Dissolve one NBT/BCIP Ready-to-Use Tablet in 10 mL ddH<sub>2</sub>O, mix, and replace NTMT buffer with 1 mL staining solution to initiate the chromogenic reaction.

**Note:** If AP substrates other than specified are used, prepare staining solution according to the manufacturer's instructions or dilute the substrate in NTMT buffer to ensure that both pH and salt concentrations are optimal for the AP enzyme.

- b. Decant reaction tubes containing staining solution and larvae into a 12-well plate.
  - c. Incubate larvae in the dark.

**Note:** The staining reaction should be monitored every 15–30 min using a stereo microscope (Figure 1D) to prevent overstaining. If no background signal develops, the staining procedure can be continued for several days. In that case, renew staining solution daily.



27. Stop the staining reaction by washing larvae 2× in PBST for 1 min each.

**Note:** Larvae can be stored in PBST for several days to weeks at 4°C.

**Optional:** When NBT/BCIP or BM-Purple substrates are used, background staining can be removed by clearing larvae in EtOH. Note that this needs to be done soon after the termination of the staining reaction as after some days the background precipitate cannot be efficiently dissolved.

**Note:** NBT/BCIP and BM-Purple result in an insoluble blue precipitate. In these cases, clearing with EtOH is recommended. However, Fast Red and INT/BCIP stains are soluble in EtOH and the background cannot be cleared.

- a. Wash in 50% EtOH-PBST for 5 min.
- b. Incubate larvae 2× in 100% EtOH for 5–15 min each.
- c. Wash in 50% EtOH-PBST for 5 min.
- d. Wash 4× in PBST for 5 min each.

**Note:** Larvae can be stored in PBST or 75% glycerol for several days at 4°C.

28. Decapitate larvae and mount trunks in 75% glycerol on a microscope slide. Cover samples with a coverslip and seal with transparent nail polish.

**Note:** Decapitation facilitates the lateral positioning and alignment of the larval zebrafish trunks.

29. Image larvae with a stereo microscope or laser scanning confocal microscope for Fast Red-stained samples.

**Note:** Fast Red produces a fluorescent red precipitate with an excitation peak at 560 nm and an emission peak at 635 nm.

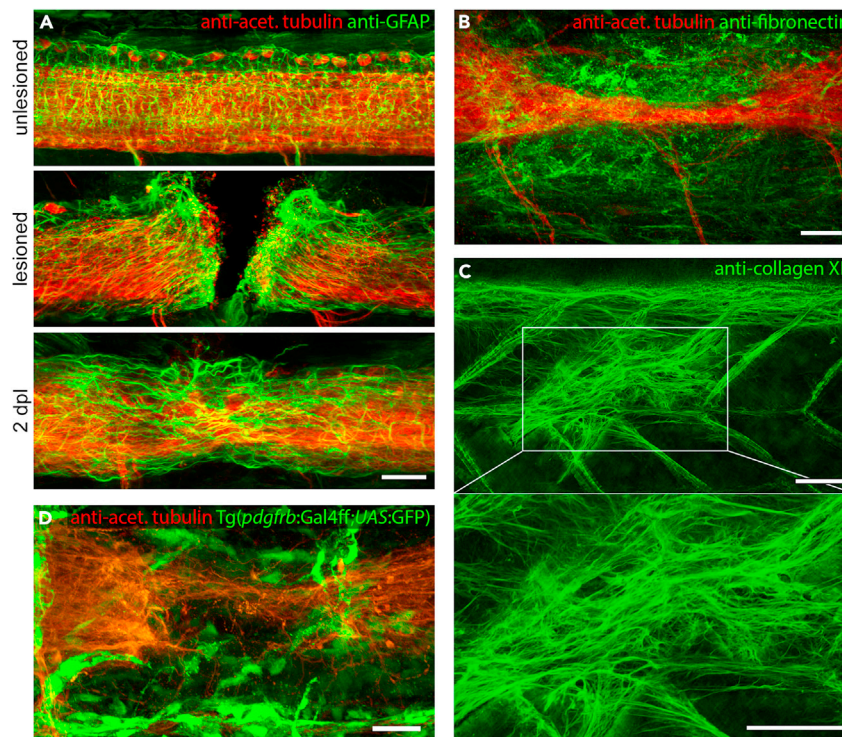
### Whole-mount immunohistochemistry

⌚ **Timing:** 6 days

The following protocol describes immunohistochemistry (IHC) to detect specific proteins in whole-mount zebrafish larvae, such as acetylated tubulin to label neurites. After spinal cord lesion (0–2 dpf), zebrafish larvae are fixed with PFA to prepare them for labeling. Fixed larvae are permeabilized and probed with antibodies of interest. This protocol enables efficient antibody penetration which can detect proteins located as deeply as in the spinal cord of 5 dpf (2 dpf) larvae and which is also sufficiently sensitive for the simultaneous detection of extracellular matrix (ECM) proteins (Figure 4B–C) (Wehner et al., 2017; Tsata et al., 2021). Another advantage of this protocol is that it preserves the signal of fluorescent proteins of reporter transgenes (Figure 4D).

Unless stated otherwise, all steps are carried out at RT. Media are drawn with a vacuum pump. Care should be taken not to aspirate tissue samples. Small volumes can be aspirated using a fine hypodermic needle ( $\leq 27\text{ G} \times \frac{3}{4}\text{''}$ ) attached to the vacuum pump.

**△ CRITICAL:** For all incubation steps  $\geq 10$  min, samples are placed in a tube rack tilted by 90°. To ensure that exact incubation times are met, set the tubes upright about 1 min before the end of the incubation period to allow samples to sink to the bottom of the tube. Gently tap the tubes to ensure that there are no samples floating on the surface before aspirating the supernatant.



**Figure 4. Representative examples of whole-mount IHC in larval zebrafish**

(A) Co-labeling of neurites (anti-acetylated tubulin; red) and astroglia-like processes (anti-GFAP; green) in the spinal cord of unlesioned larvae (top panel), immediately after incision lesion (middle panel) and at 2 dpl (bottom panel). Continuity of axonal labeling between rostral and caudal spinal cord stumps is restored within 2 dpl (bottom panel). (B) Co-labeling of neurites (anti-acetylated tubulin; red) and fibronectin matrix (anti-Fibronectin; green) in the lesion site at 1 dpl (perforation lesion). (C) Labeling of lesion-induced collagen type XII matrix deposition at 2 dpl (perforation lesion). (D) Labeling of neurites with anti-acetylated tubulin immunohistochemistry (red) preserves the GFP signal of the GFP reporter in *Tg(pdgfrb:Gal4ff;UAS:EGFP)* transgenic animals. GFP<sup>+</sup> cells accumulate in the lesion site at 1 dpl (perforation lesion). (A–D) Images shown are maximum intensity projections of the unlesioned trunk or the lesion site at indicated timepoints after lesion (lateral view; rostral is left, dorsal is up). Scale bars: 50  $\mu$ m (C) and 25  $\mu$ m (A, B, D).

30. Terminally anesthetize larvae by adding an overdose of MS-222 to E3 medium (final concentration of  $\geq 0.1\%$ ).
31. Transfer larvae to a 2 mL reaction tube using a transfer pipette.
32. Fix larvae in 4% PFA for exactly 1 h.

**△ CRITICAL:** Do not fix longer than 1 h, otherwise antibody penetration may be impaired.

33. Wash 2 $\times$  in PBST for 1 min each.
34. Dehydrate larvae stepwise by successive incubation in 25% MeOH-PBST, 50% MeOH-PBST, 75% MeOH-PBST and 100% MeOH for 5 min each.

**Note:** Samples can be stored in 100% MeOH at  $-20^{\circ}\text{C}$  for several months.

**△ CRITICAL:** Methanol is acutely toxic and flammable. It causes damage to organs in direct contact, or if swallowed or inhaled. Wear protective gloves, protective clothing, eye and face protection.

35. Transfer larvae to a plastic petri dish (35 mm in diameter) filled with 100% MeOH. Remove heads and tails using micro scissors under visual control (stereo microscope). This will improve penetration of the antibodies.
36. Transfer the trunk tissue containing the lesion site to a new 1.5 mL reaction tube using a glass Pasteur pipette with rubber bulb and incubate samples in 100% MeOH at  $-20^{\circ}\text{C}$  for 14–18 h.
37. Stepwise rehydrate samples by successive incubation in 75% MeOH-PBST, 50% MeOH-PBST, 25% MeOH-PBST and PBST for 5 min each.
38. Wash 2× in ddH<sub>2</sub>O for 5 min each.

△ **CRITICAL:** Remove as much ddH<sub>2</sub>O as possible to avoid dilution of acetone in the next step.

39. For permeabilization, incubate samples in 100% acetone (pre-chilled to  $-20^{\circ}\text{C}$ ) at  $-20^{\circ}\text{C}$  for exactly 10 min.
40. Aspirate acetone and wash samples in ddH<sub>2</sub>O for 5 min.

△ **CRITICAL:** Remove as much acetone as possible to reduce the sticking of tissue to the reaction tube wall in the following steps.

41. Wash 2× in PBST for 5 min each.

△ **CRITICAL:** Samples will stick to the tube wall. To avoid sample loss, aspirate the supernatant using a transfer pipette pre-coated with PBST. Discard the supernatant into 50 mL conical centrifuge tubes filled with PBST. Accidentally-withdrawn samples can thereby be collected and returned to the reaction tube.

42. For further permeabilization, incubate samples in PBST containing 5–7.5  $\mu\text{g}/\text{mL}$  Proteinase K for 15 min.

△ **CRITICAL:** Due to batch-to-batch variations of Proteinase K activity, its concentration and incubation time needs to be tested and adjusted for each batch to avoid over or under digestion of the samples. As a starting point, we recommend 15 min incubation of 5  $\mu\text{g}/\text{mL}$  or 7.5  $\mu\text{g}/\text{mL}$  to permeabilize 3–4 dpf (0–1 dpl) or 5 dpf (2 dpl) zebrafish larvae, respectively.

43. Wash samples 2× in PBST for  $\leq 1$  min each.
44. Re-fix samples by incubating in 4% PFA for exactly 15 min.

△ **CRITICAL:** Over fixation may mask epitopes and/or reduce antibody penetration.

45. Wash samples 3× in PBTx for 10 min each on a see-saw rocking shaker with low agitation at  $\sim 10$  rpm.
46. Incubate in IHC blocking solution for a minimum of 1 h on a see-saw rocking shaker with low agitation at  $\sim 10$  rpm.
47. Incubate in IHC blocking solution containing primary antibody at  $4^{\circ}\text{C}$  for  $\geq 3$  nights on a see-saw rocking shaker with low agitation at  $\sim 10$  rpm.

**Note:** The working dilution needs to be determined for each antibody. We routinely use antibodies to detect endogenous proteins at a dilution of 1:300. See [Table 2](#) for a list of primary antibodies that have been successfully used with the described protocol.

48. To remove unbound antibody, wash samples 8× in PBTx for 15 min each on a see-saw rocking shaker with low agitation at  $\sim 10$  rpm.

**Table 2. Primary antibodies successfully used with the described protocol**

Antibody	Source	Identifier	Working dilution	Used in
Rabbit anti-Fibronectin	Sigma-Aldrich	Cat#F3648; RRID: AB_476976	1:300	Tsata et al. (2021) Wehner et al. (2017)
Rabbit anti-Collagen XII	Bader et al. (2009)	n/a	1:250	Tsata et al. (2021) Wehner et al. (2017)
Rabbit anti-Tenascin C	US Biological	Cat#T2550-23; RRID: AB_2271850	1:300	Tsata et al. (2021)
Rabbit anti-Collagen I	Abcam	Cat#ab23730; RRID: AB_447645	1:300	Wehner et al. (2017) Tsarouchas et al. (2018)
Mouse anti-Tubulin, acetylated	Sigma-Aldrich	Cat#T6793; RRID: AB477585	1:300	Tsata et al. (2021) Wehner et al. (2017) Tsarouchas et al. (2018)
Rabbit anti-Glial Fibrillary Acidic Protein	Agilent	Cat#Z0334; RRID: AB_10013382	1:300	Wehner et al. (2017)
Chicken anti-GFP	Abcam	Cat#ab13790; RRID: AB_300798	1:500	Tsata et al. (2021)

49. Incubate in IHC blocking solution containing appropriate fluorescent secondary antibody at 4°C for 2 nights on a see-saw rocking shaker with low agitation at ~10 rpm.

**Note:** Protect the samples from light in subsequent steps.

50. To remove unbound antibody, wash 6 × in PBTx for 10 min each on a see-saw rocking shaker with low agitation at ~10 rpm.
51. Wash samples 2 × in PBS for 1 min each to dilute PBTx.
52. Mount larvae in 75% glycerol or commercially available antifade mounts on a microscope slide, cover with a cover slip, and seal with transparent nail polish.
53. Image samples with a laser scanning confocal microscope for high resolution.

## EXPECTED OUTCOMES

This protocol describes two different procedures to transect the larval zebrafish spinal cord. Acutely lesioned zebrafish larvae are initially paralyzed caudal to the lesion site (absence of touch-evoked escape response) (Ohnmacht et al., 2016; Tsarouchas et al., 2018) and exhibit anatomical discontinuity of the spinal cord (i.e., lack of neuronal labeling in the lesion site; Figure 4A and Wehner et al., 2018; Wehner et al., 2017). However, axonal regrowth (axonal bridging of the lesion site) and recovery of swimming function is observed within two days after injury (Ohnmacht et al., 2016; Wehner et al., 2017; Tsarouchas et al., 2018; Tsata et al., 2021). Lesioned animals should exhibit a high baseline of survival (> 95%) and a high regeneration rate; i.e., typically ≥ 80% of animals show continuity of axonal labeling between severed spinal cord ends at 2 dpl. Axonal regrowth can be visualized in live animals using transgenic fluorescence reporter zebrafish lines (e.g., Tg(*elav3*:*rasmKate2*) (Tsata et al., 2021)), and in whole-mount histological preparations using anti-acetylated tubulin IHC (see Figure 4 for sample images and Wehner et al., 2017; Tsarouchas et al., 2018; Tsata et al., 2021).

The described ISH protocol allows efficient RNA probe penetration in 5 dpf (2 dpl) whole-mount zebrafish larvae and thus, enables the observation of both homeostatic and regeneration-associated gene expression at high spatial resolution. The expected outcome is the specific and clear labeling of mRNA expression, which is readily identifiable using a stereo microscope. There should be low background staining and a high signal-to-noise ratio. Injury-induced gene expression should be confined to the lesion site or signal intensity in the lesion site should be higher than in adjacent unlesioned trunk tissue (see Figure 3 for sample images). Overall, if the presented protocol is followed, genes involved in spinal cord regeneration can be identified.

The described whole-mount IHC protocol provides clear and homogeneous labeling of neurites (anti-acetylated tubulin immunohistochemistry) and, if applicable, concomitant visualization of the protein of interest, including ECM proteins and GFAP to label astroglia-like cells (see [Figures 4A–4C](#) for sample images and [Wehner et al., 2017](#)). There should be low background fluorescence and a high signal-to-noise ratio. Furthermore, endogenous fluorescence protein signals should be preserved by the protocol and readily detectable (see [Figure 4D](#) for sample image). Overall, if the described protocol is followed, regeneration of axons, as well as their interaction with different target cells and ECM proteins in the lesion environment, can be studied.

## LIMITATIONS

In this protocol we describe two procedures to mechanically transect the spinal cord in zebrafish larvae. However, to perform these surgeries in a consistent and reproducible manner, training is needed. To this end, we recommend using transgenic reporter lines with fluorescently labeled neurons, such as *Tg(elavl3:rasmKate2)* ([Tsata et al., 2021](#)), which will enable *in vivo* examination of the extent of spinal cord transection and successful axonal regeneration.

When using Fast Red substrate for fluorescent visualization of transcripts in the described ISH protocol, weakly expressed genes cannot be detected at all or only with high background noise. In this case, we suggest using conventional chromogenic substrates that produce dark blue stains (e.g., NBT/BCIP). Alternatively, amplification methods can be used to increase the sensitivity of the fluorescence ISH signal, such as tyramide signal amplification ([Brend and Holley, 2009](#); [Lauter et al., 2014](#)), which has successfully been applied to both the regenerating zebrafish spinal cord and fin ([Wehner et al., 2014, 2017](#)).

The described IHC protocol allows the robust detection of specific proteins in whole-mount zebrafish larvae. However, only a limited number of zebrafish-specific antibodies are available. Antibodies that have not previously been used in zebrafish need to be validated for antigen specificity to exclude non-specific binding. Furthermore, permeabilization of the tissue requires careful optimization to both achieve sufficient antibody penetration and minimize the risk of over digestion of sensitive epitopes, such as ECM proteins. Hence, when co-labeling neurites and ECM proteins, the immunoreactivity of the latter could be an underestimate of its true *in vivo* abundance.

## TROUBLESHOOTING

### Problem 1

Trunk of larva does not settle in a lateral position or side-slips while lesioning (steps 3 and 4).

#### Potential solution

Ensure that excess E3 medium has been sufficiently removed prior to surgery, using the aspiration pipette (steps 3b and 4b). When the surgery plates are freshly prepared, the wettability of the surface is poor, which impairs the removal of excess E3 medium without aspirating larvae. Ensure that the surface has been roughened sufficiently and keep reusing surgery plates, as they improve over time ([before you begin](#) step 3).

### Problem 2

High mortality rate of lesioned larvae.

#### Potential solution

A possible reason for an increased mortality rate of lesioned larvae can be desiccation. Leave sufficient volume of E3 medium on the surgery plate to keep larvae hydrated and quickly return the larvae to the recovery dish after surgery (steps 3 and 4). We suggest starting with lesioning only a few animals ( $\leq 3$ ) at a time. This number can be increased to a recommended maximum of ten when surgery skills are advanced and less time is needed per animal.

High mortality among lesioned animals could also be due to oversized perforation lesions. Ensure that only the very tip of the micro scalpel is used to induce the lesion and that the notochord is not injured (step 3c).

Ensure that the MS-222 concentration for anesthesia does not exceed the recommended concentration (step 1).

### Problem 3

Low regeneration rate: i.e., low proportion of animals that exhibit axonal bridging of the lesion site at 2 dpl.

#### Potential solution

Check whether the notochord was injured using a stereo microscope (Figure 2H). Axonal regeneration is significantly impaired when the notochord is extensively damaged. Ensure that the notochord is left intact when lesioning the larvae (steps 3c and 4c).

### Problem 4

Weak or absent ISH signal.

#### Potential solution

Absent ISH signal: RNA probe might have been synthesized in sense orientation, which precludes hybridization of the probe to the mRNA. Verify polymerase promoter position at the 3' end of the probe template.

Absent ISH signal: Probe might not match conjugate. Check that the labeled RNA probes match the conjugate; digoxigenin-labeled probes can only be detected with anti-digoxigenin antibodies (step 22).

Absent ISH signal: Enzyme conjugate (AP) may not be active. Verify activity of AP enzyme by mixing antibody with substrate, which should readily result in a color change.

Low ISH signal: Low RNA probe penetration due to insufficient permeabilization. Due to batch-to-batch variations of Proteinase K activity, it is critical to adjust the concentration and/or incubation time of Proteinase K (step 12).

Low ISH signal: Low expression of gene of interest. Consider amplification steps to enhance signal, such as tyramide signal amplification (Brend and Holley, 2009; Lauter et al., 2014).

### Problem 5

High background signal after ISH.

#### Potential solution

The concentration of RNA probe or conjugate might be too high. Try decreasing the probe and/or antibody concentration (steps 17 and 22).

Excessive staining duration. We recommend monitoring the staining reaction by microscopy to stop the reaction as the background develops (steps 26 and 27).

### Problem 6

Weak or incomplete fluorescence signal after IHC.

### Potential solution

Enhance antibody penetration by adjusting permeabilization. Increase concentration and/or incubation time with Proteinase K. However, a low fluorescence signal can also be the result of over digestion. In this case, Proteinase K concentration and/or incubation time should be reduced (step 42).

Ensure that the microscope is equipped with the correct light source and filter set for the chosen fluorophore (step 53).

### Problem 7

High background fluorescence signal after IHC.

### Potential solution

Reduce primary and/or secondary antibody concentration and/or increase the blocking incubation time. Additionally, the number of washes can also be increased (steps 46–50).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel Wehner ([daniel.wehner@mpl.mpg.de](mailto:daniel.wehner@mpl.mpg.de)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate any new code or analyze any dataset.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.101093>.

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## AUTHOR CONTRIBUTIONS

Conceptualization: D.W.; Methodology: D.W.; Investigation: N.J., J.K., D.W.; Writing – Original Draft: N.J., J.K., D.W.; Writing – Review & Editing: N.J., J.K., D.W.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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