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# Spinal Cord Transection In *Xenopus laevis* Tadpoles

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## Abstract

Spinal cord injury (SCI) is a permanent affliction, which affects the central nervous system (CNS) motor and sensory nerves, resulting in paralysis beneath the injury site. To date, there is no functional recovery therapy for SCI, and there is a lack of clarity regarding the many complexes and dynamic events occurring after SCI. Many non-mammalian organisms can regenerate after severe SCI, such as teleost fishes, urodele amphibians, and larval stages of anuran amphibians, including *Xenopus laevis* tadpoles. These are bona fide model organisms to study and understand the response to SCI and the mechanisms underlying successful regenerative processes. This type of research can lead to the identification of potential targets for SCI therapeutic intervention. This article describes how to perform *Xenopus laevis* tadpole spinal cord transection, including husbandry, surgery, postsurgery care, and functional test evaluation. This injury method can be applied for elucidating the different steps of spinal cord regeneration by studying the cellular, molecular, and genetic mechanisms, as well as histological and functional evolution after SCI and during spinal cord regeneration.

## Introduction

Spinal cord injury (SCI) is an affliction that affects approximately 250,000-500,000 people worldwide every year[1]. In addition to this high prevalence, SCI affects sensory and motor nerves, generating paralysis beneath the injury site and disconnection of some internal organs from the control of the CNS. The spinal cord, a part of the CNS, cannot regenerate, and due to the complexity of the affliction and the lack of complete understanding of all the involved processes, there are still no efficient therapies allowing functional recovery.

Non-mammalian organisms, such as teleost fishes, urodele amphibians, and larval stages of anuran amphibians, which can regenerate the spinal cord after severe SCI[2,3,4], are excellent model organisms for studying the processes that govern a successful regenerative event and understanding the failure of mammalian regeneration. This understanding is

of great interest as it could provide original insights to develop new therapeutic targets and possible therapies for SCI.

The anuran frog, *Xenopus laevis,* is an excellent model organism to study SCI. It has excellent regenerative capacities during the tadpole stages, which are progressively lost during metamorphosis, allowing experimentation in the regenerative and nonregenerative stages[3,5]. The established injury method for studying SCI in *Xenopus laevis* tadpoles consists of tail amputation, where the entire tail is removed, including tissues such as muscle, notochord, and spinal cord[6]. This approach has been instrumental in the understanding of general mechanisms of regenerative processes[4,7,8,9,10].

As tail amputation involves multiple tissues in addition to the spinal cord, which is different from what happens after human SCI, a more relevant injury paradigm is needed for the study of SCI. We have relied on studies used in the past[11] for generating comprehensive descriptions of injury paradigms[5,12,13,14] and different methods for the study of SCI[12,13,14,15,16,17,18]. After spinal cord transection, the caudal portion of the spinal cord can be isolated for RNA and protein expression and high-throughput analyses[14,19,20,21]. Additionally, intracelomic injections of drugs and small molecules, as well as electroporation of cDNA, RNA, or morpholinos, before or after spinal cord transection, allow the study of the effects of these molecules in the prevention or treatment of SCI or of specific events occurring after SCI and spinal cord regeneration[13,14]. Further, injury evolution and the regenerative processes can be studied at different timings after injury using biochemical, molecular, histological, and functional approaches[12,13,14,17,19,20,21,22,23].

Finally, all the aforementioned techniques can be used in non-regenerative stages, highlighting one of the most important advantages of using *Xenopus laevis* as a model organism to study SCI, the comparative studies of regenerative and non-regenerative mechanisms in the same species[13,19,20,21,22]. This paper presents a protocol for *Xenopus laevis* tadpole spinal cord transection, starting with the staging and selection of regenerative Nieuwkoop and Faber (NF) stage 50 tadpoles. This is followed by the description of the procedures for spinal cord surgery to produce sham and transected animals, postsurgical care, and finally the analysis of functional recovery by the measurement of free tadpole swimming distance.

## Protocol

This protocol provides enough information to successfully perform spinal cord transection. Of note, there are excellent detailed protocols of these techniques published elsewhere[14], which can complement the one presented here. All animal procedures have been approved by the Committee on Bioethics and Biosafety from the Faculty of Biological Sciences, Pontificia Universidad Católica de Chile.

### 1. Natural mating of frogs

1. Three to five days prior to mating, subcutaneously preinject male and female frogs with 50 units of human chorionic gonadotropin (hCG). Use the "iron claw" technique for restraining the frogs; as the frogs are slippery, use a net to surround the frog if necessary. Insert the tip of a 26 G x ½" needle posterior to the lateral line, pushing it dorsally to a depth of 1 cm, between the skin and the muscle.
2. Before mating, inject the male with 300 units and the female with 700 units of hCG.

3. For mating to occur, place the male and the female in 2 L of 0.1x Barth solution immediately after chilling the solution at 4 °C for 15 min to resemble spring conditions and leave overnight at 18 °C.

4. Sixteen hours later, carefully collect the embryos with the help of a plastic Pasteur pipette, with the tip cut off, and place them in 10 cm diameter Petri dishes. Remove the embryonic jelly coat by incubating the embryos with 25 mL of 2% cysteine in distilled water (pH 7.8; ensure the solution covers the embryos) for 5 min with slight agitation. Wash 3 times with distilled water and 3 times with 0.1x Barth solution (8.9 mM NaCl; 102 µM KCl; 238.1 µM $NaHCO_3$; 1 mM 4-(2- hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES); 81.14 µM $MgSO_4$; 33.88 µM $Ca(NO_3)_2$; 40.81 µM $CaCl_2$, pH 7.6).

5. Select healthy embryos that have a brownish color and symmetrically dividing blastomeres. Place the embryos in 10 cm diameter Petri dishes with 50 mL of 0.1x Barth solution at a density of no more than 100 embryos per dish.

## 2. Husbandry

1. During the first week, maintain the embryos at 18 °C until they get off the vitelline sac. During this time, change the Barth solution every day, and remove whitish dead embryos and tadpoles presenting any visible anatomical alteration or tadpoles without any swimming movement.

2. After the first week, transfer tadpoles to chlorine-free water in plastic tanks at a density of 10 animals per liter. Grow tadpoles at 20-21 °C with a 12-h light/12-h dark cycle, with oxygen stones available in each tank to aerate the water and fed once a day with 0.5 mg per animal. Replace water once a week and check for accumulated waste and dead animals daily[24].

## 3. Staging

1. Three to four weeks after fertilization, place the animals in a Petri dish; then, one by one, check the morphology and appearance of forelimbs and hindlimbs. If necessary, anesthetize the animals by placing the animals in a Petri dish with 50 mL of 0.02% tricaine mesylate in 0.1x Barth solution for better manipulation. After no more than 2 min, place the animals in 0.1x Barth solution for recovery from the anesthesia.

2. Look for the following anatomical characteristics of stage 50 animals[25]: forelimbs that are just appearing and are spherical (**Figure 1**); hindlimbs that are protruding and are spherical (**Figure 1**).
NOTE: Animals from stages 49 to 51 can be used for this procedure (**Figure 1**); for more information about stages, refer to Nieuwkoop and Faber's Normal Table of *Xenopus laevis*[25].

## 4. Surgery: spinal cord transection and sham-operated animals

1. Anesthetize stage 50 tadpoles by placing them in a Petri dish with 50 mL of 0.02% tricaine mesylate in 0.1x Barth solution for 2 min.

2. With the help of a tablespoon and forceps, place the tadpole, dorsal side-up, on a wet piece of gauze in the upper half of a glass Petri dish.

3. Perform an incision of the skin and dorsal muscles at the mid-thoracic level (**Figure 2A, B**) using microdissection spring scissors.

1. For control sham animals, ensure that the incision size is only ~0.2 mm (**Figure 2C**); do not damage the spinal cord (**Figure 2D,D´**).
2. For transected animals, perform a second incision of ~0.2 mm (**Figure 2C**) to fully transect the spinal cord (**Figure 2E,E´**).

### 5. Postsurgery care

1. After surgery, transfer the tadpoles to a tank containing 0.5 L of 0.1x Barth solution with 1x Penicillin-streptomycin, at a density of 10-12 animals per tank. Maintain the transected and control sham animals in separate tanks.
   NOTE: Tadpoles will recover from the anesthesia in a couple of minutes.
2. Maintain the tadpoles with aeration at a temperature of 20-21 °C.
3. Change the Barth solution with antibiotics every other day until the end of the experiment.
4. Start feeding the animals one day after surgery, once per day.
5. Eliminate dead animals.

### 6. Swimming assay

1. Obtain a box with LED illumination from the inside, covered with a transparent polystyrene sheet, which allows the light to pass through.
2. Install a camera over the LED box.
3. Place a 15 cm diameter Petri dish on top of the box, filled with 100 mL of 0.1x Barth solution.
4. One day post transection, place a tadpole in the Petri dish and leave for a 5 min adaptation period.
5. After adaptation, start video-tracking the free-swimming behavior using the referenced software (see the **Table of Materials**) for 5 min.
6. After the video is completed, transfer the tadpole back to its tank.
7. Repeat the video tracking 5, 10, 15, and 20 days post transection (**Figure 3**).

### 7. Bioethical considerations

NOTE: The mortality of animals after sham surgery and transection is 13% and 30%, respectively. Additionally, a minimum of 15-20 animals per group is necessary for statistical analysis. Therefore, start with 23 sham and 26 transected animals.

1. Anesthetize the animals with 0.02% tricaine mesylate for 2 min to assure reduction in neuronal and motor activity and pain before surgery.
2. After surgery, check the animals for recovery from anesthesia. Additionally, feed and check the animals daily.
3. After finishing the swimming assay, sacrifice the animals with an overdose of tricaine mesylate (1% tricaine mesylate prepared in 30 mM sodium bicarbonate solution).

## Representative Results

The protocol described herein allows the study of spinal cord regeneration in *Xenopus laevis*. The effects of specific pharmacological treatments and the contribution of specific gene expression in spinal cord regeneration can be evaluated by measuring their effects on swimming recovery. The total swimming distance is plotted against the days after injury

to compare control and treated animals at a specific time point or over a specified period. The recovery of motor function through time is exemplified in **Figure 3**, showing the swimming distance at 5, 10, 15, and 20 days post transection. At 5 days post transection, animals swam an average of 0.7 m in 5 min, showing a reduced swimming capacity. This capacity increased with the passing days, as an average of 2.1 and 3.1 m/5 min was observed after 10 and 15 days post transection, respectively, and complete recovery of swimming capacities was observed at 20 days post transection, with an average of 5.7 m/5 min.

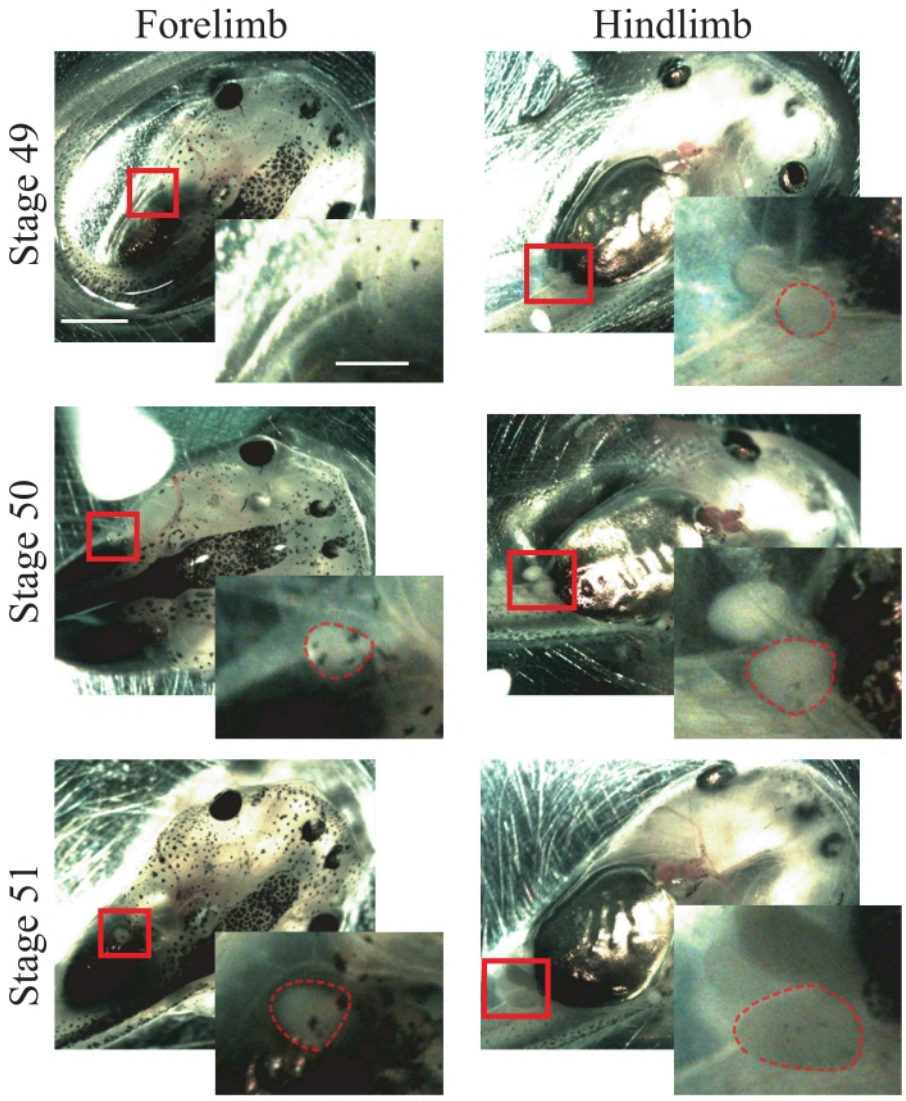


**Figure 1: Xenopus tadpole staging.** Representative images of stages 49-51, showing fore- and hindlimbs for animal staging reference. Scale bars = 2 mm. Magnifications of the boxed region are shown in the lower-right of each image.Scale bars = 1 mm. In stage 49, forelimbs are not observed, while hindlimbs are just appearing, showing a spherical shape. Stage 50 presents forelimbs that are just appearing, showing a spherical shape and hindlimbs protruding with a spherical shape. In stage 51, forelimbs present a protruding spherical shape and hindlimbs a protruding elongated shape. Dashed outlines show fore- and hindlimbs. Please click here to view a larger version of this figure.

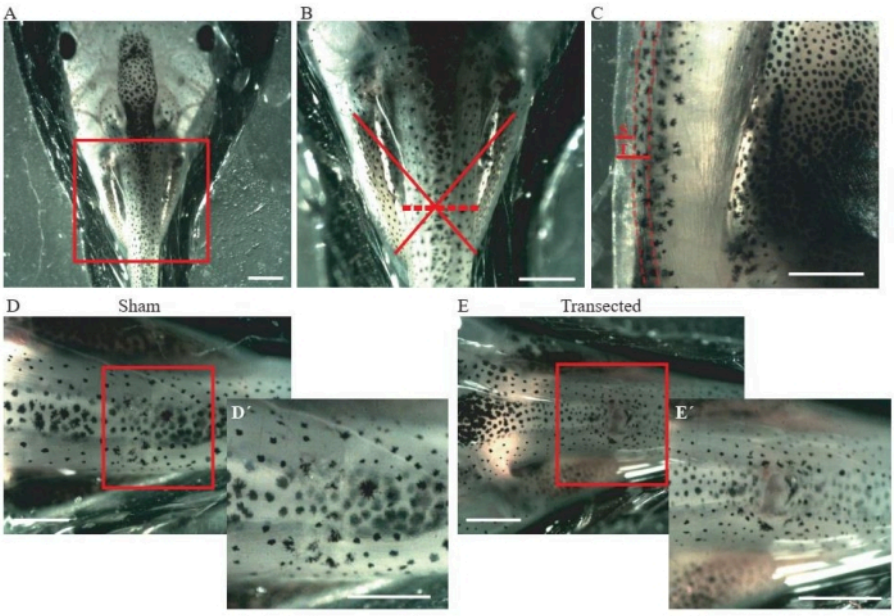


**Figure 2: Spinal cord transection.** (**A**) Representative image showing the correct positioning of the animal, dorsal side up, for performing the surgery. Scale bar = 2 mm. (**B**) Magnification of **A** shows the location and extent of injury. The red cross shows the exact location of the injury site at the thoracic level of the spinal cord, and the dashed line shows the extent of the injury. Scale bar = 1 mm. (**C)** Representative image showing a lateral view of the thoracic level of the spinal cord. The extension of the sham incision and transection are shown. Dashed lines delineate the limits of the spinal cord. Scale bar = 1 mm. (**D**) Representative image showing a sham animal with an intact spinal cord. Scale bars = 1 mm. (**E)** Representative image showing a transected animal with an interrupted spinal cord. Scale bars = 1 mm. Magnifications of the boxed region are shown in the lower-right of each image (D´ and E´).Scale bars = 1 mm. Abbreviations: S = sham incision; T = transection.

Please click here to view a larger version of this figure.

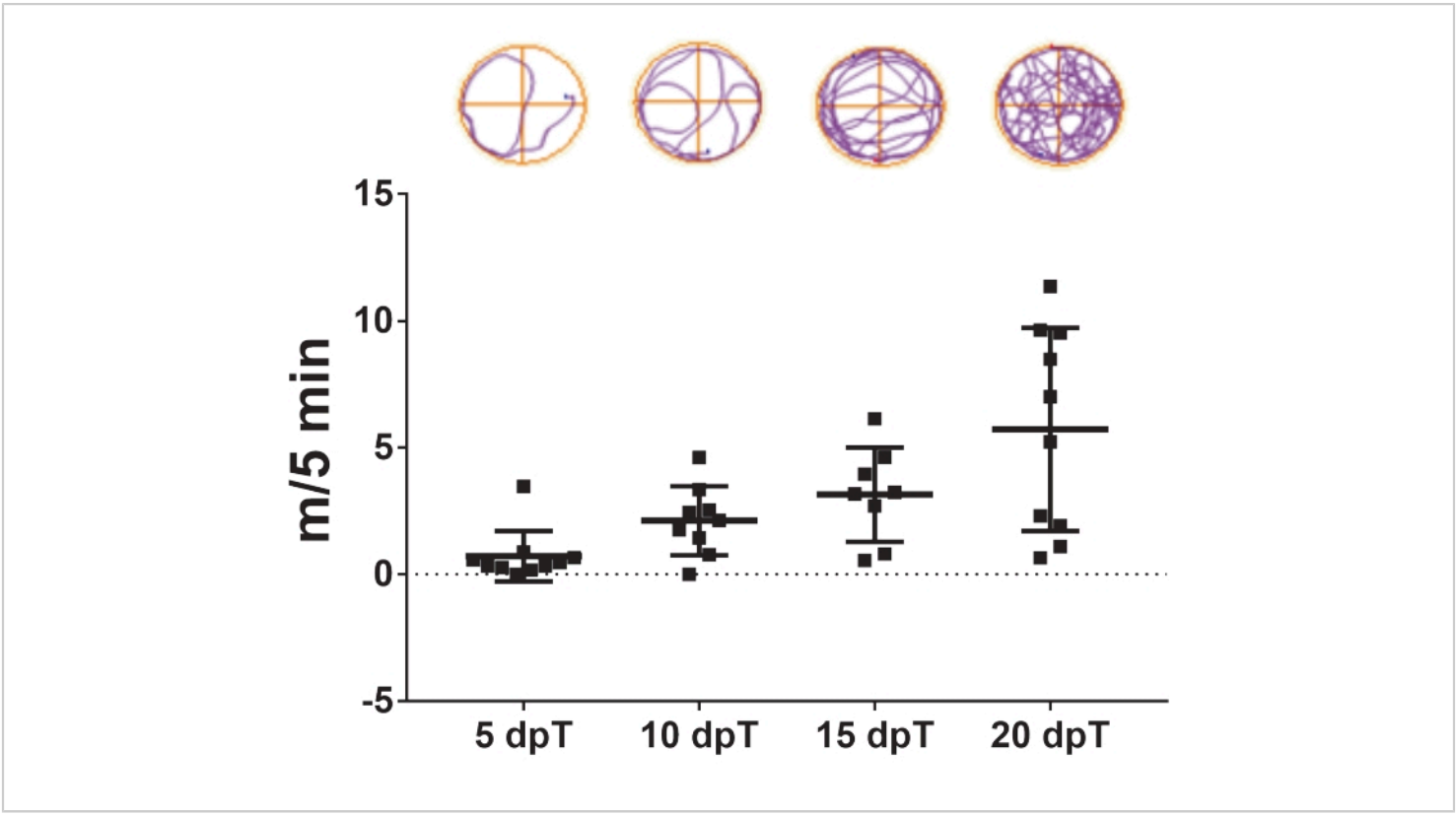


**Figure 3: Swimming function recovery over time.** Representative dot plot of the swimming distance covered by transected animals in 5 min at 5, 10, 15, and 20 days post transection. Samples of swimming trajectories are shown on top. Data presented as mean ± SEM from 10 tadpoles. Abbreviations: dpT = days post transection; SEM = standard error of the mean. Please click here to view a larger version of this figure.

## Discussion

The protocol described herein is an excellent method to perform SCI and evaluate functional recovery. For reproducibility, it is essential to grow healthy tadpoles and choose animals that are similar in size. Lack of proper feeding generates nutrient stress, which results in poor regenerative capacities[26]; therefore, special attention should be paid to tadpole feeding. As tadpoles reach stage 50 after 3-4 weeks, they can be reared at higher temperatures to accelerate the growth process, 18-25 °C being optimal[27]. Water quality is important, as animals are sensitive to water conditions and chemical products. The optimal water conditions include using carbon filtered, chlorine-free water with the following parameters: pH (6.5-7.5), chloride (<0.02 mg/L), conductivity of water (1.0 mS/cm ± 0.1 units), copper (<0.3 mg/L); carbonate hardness (KH: 5-10 dKH); general hardness (GH: 6-16 dGH); nitrate ($NO_3$: <20 mg/L); and nitrite ($NO_2$: <0.1 mg/L)[14,27,28]. Additionally, to avoid contamination, plastic tanks should be cleaned once a week for rearing animals or every other day after surgery by washing thoroughly with chloride-free water and a sponge; detergent must be avoided.

For a better survival rate after surgery, tadpoles must not be exposed to anesthesia for long periods (no longer than 2 min). Moreover, it is recommended to anesthetize one tadpole at a

time. As the animals need to stay hydrated, keep the animals immersed in solution all the time before and after surgery, and pour the solution with a spoon on top of the tadpole before beginning the surgery. Ensure that the damage is extensive enough to cover the whole spinal cord but not too extensive as it can induce poor functional recovery or death. If the notochord is damaged, the animal will be bent, and the functional recovery will be affected. If the damage extends beyond the notochord, the probability of death increases[14]. During the swimming assay, recording is considered correct if the software identifies each animal with a blue shadow; otherwise, the recording should be repeated. It is important to avoid movement and air or light changes during the recording process to prevent recording mistakes.

There are still many open questions about the cellular and molecular mechanisms underlying spinal cord damage and regeneration. The protocol described in this work can be used to study the contribution of different cellular events, gene expression, and treatments on functional recovery, determined by measuring swimming capacities. Additionally, many other techniques can be applied to the operated animals. The spinal cord can be isolated to perform protein and/or mRNA extraction[14] to study protein and gene expression profiles after damage and treatment[19,20]. This surgery has also been the basis for studying the spinal cord cellular response[22] and the behavior of neural stem progenitor cells[12,13,22] after spinal cord injury. Signaling cascades involved in spinal cord regeneration have also been studied using the spinal cord damage paradigm described herein[23]. In summary, the protocol described here is an excellent model to study spinal cord injury and regeneration and has been used for many studies that have contributed to the existing knowledge about the subject.

## Disclosures

The authors have no conflicts of interest to declare.

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