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TITLE:

Contusion Spinal Cord Injury via a Microsurgical Laminectomy in the Regenerative Axolotl

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spinal cord injury, trauma, regeneration, axolotl, microsurgery, ultrasonography

SUMMARY:
This manuscript presents protocols for surgically inflicting controlled blunt and sharp spinal cord injuries to a regenerative axolotl (*Ambystoma mexicanum*).

ABSTRACT:
The purpose of this study is to establish a standardized and reproducible regenerative blunt spinal cord injury model in the axolotl (*Ambystoma mexicanum*). Most clinical spinal cord injuries occur as high energy blunt traumas, inducing contusion injuries. However, most studies in the axolotl spinal cord have been conducted with sharp traumas. Hence, this study aims to produce a more clinically relevant regenerative model. Due to their impressive ability to regenerate almost any tissue, axolotls are widely used as models in regenerative studies and have been used extensively in spinal cord injury (SCI) studies. In this protocol, the axolotls are anesthetized by submersion in a benzocaine solution. Under the microscope, an angular incision is made bilaterally at a level just caudal to the hind limbs. From this incision, it is possible to dissect and expose the spinous processes. Using forceps and scissors, a two-level laminectomy is performed, exposing the spinal cord. A custom trauma device consisting of a falling rod in a cylinder is constructed, and this device is used to induce a contusion injury to the spinal cord. The incisions are then sutured, and the animal recovers from anesthesia. The surgical approach is successful in exposing the spinal cord. The trauma mechanism can produce contusion injuries to the spinal cord, as confirmed by histology, MRI, and neurological examination. Finally, the spinal cord regenerates from the injury. The critical step of the protocol is removing the spinous processes without inflicting damage to the spinal cord. This step requires training to ensure a safe procedure. Furthermore, wound closure is highly dependent on not inflicting unnecessary

damage to the skin during incision. The protocol was performed in a randomized study of 12 animals.

INTRODUCTION:

The overall goal of this study was to establish a controlled and reproducible microsurgical method for inflicting blunt and sharp SCI to the axolotl (*Ambystoma mexicanum*), producing a regenerative spinal cord injury model.

SCI is a severe condition that, depending on the level and extent, inflicts neurological disability to the extremities along with impaired bladder and bowel control[1-3]. Most SCI are the result of high energy blunt trauma such as traffic accidents and falls[4,5]. Sharp injuries are very rare. Therefore, the most common macroscopic injury type is contusions.

The mammalian central nervous system (CNS) is a non-regenerative tissue, hence no restoration of neurological tissue following SCI is seen[6-8]. On the other hand, some animals have an intriguing ability to regenerate tissues, including CNS tissue. One of these animals is the axolotl. It is widely used in studies of regenerative biology and is of interest in spinal cord regeneration, because it is a vertebrate[9-12].

Most SCI studies in the axolotl are performed as either amputation of the entire tail or ablation of a larger part of the spinal cord[9-12]. Recently, a new study was published on blunt injuries[13] that mimics clinical situations better. Whereas complete appendage amputation in the axolotl results in full regeneration, some non-amputation−based regenerative phenomena are dependent on the critical size defect (CSD)[14-15]. This means that injuries exceeding a critical threshold are not regenerated. To develop a regenerative model with a higher clinical translational value, this study investigated whether a 2 mm blunt trauma would exceed the CSD limit.

This method is relevant for researchers working on spinal cord regeneration in small animal models, especially in the axolotl. Furthermore, it may be of more general interest, because it exhibits a way of using standard laboratory equipment to develop a blunt trauma mechanism that is suitable for use in small animals in general.

PROTOCOL:

All applicable institutional and governmental regulations concerning the ethical use of animals were followed during this study. The study was conducted under the approval id: 2015-15-0201-0061 by the Danish Animal Experiment Inspectorate. Animals were Mexican axolotls (*Ambystoma mexicanum*, mean body mass ± STD: 12.12 g ± 1.25 g).

1. Preparation

1.1. Prepare axolotl for anesthesia.

1.1.1. Use high quality non-chemically treated tap water. If unavailable, use 40% Holtfreter's solution.

1.1.2. Dissolve 200 mg of ethyl 4-aminobenzoate (benzocaine) in 3 mL of acetone. Dissolve this solution in 1 L of tap water or 40% Holtfreter's solution.

1.2. Use a standard Petri dish (100 mm in diameter) placed under a stereo microscope as a surgical table. Place a surgical textile cloth on the Petri dish.

NOTE: Using a Petri dish as a surgical area enables moving and rotation of the animal without touching it, ensuring spinal stability during surgery.

1.3. Prepare all sterile microsurgical instruments (i.e., scissors and anatomical forceps).

2. Anesthesia

2.1. Place the axolotl in a container with benzocaine solution for approximately 45 min to ensure deep and stable anesthesia.

NOTE: The given concentration of benzocaine will cause anesthesia in all sizes of axolotls.

2.2. Check for signs of general anesthesia within 30-45 min. These include a complete lack of gill movements, righting reflex, or response to either tactile or painful stimuli (gentle pinching of toe web).

2.3. To maintain anesthesia, wrap the animals in paper towels wetted in the anesthetic solution. Wet these regularly with this solution during the surgical procedure to ensure that the skin and gills are kept moist.

2.4. Recover the animal after the surgery by placing it in a container containing fresh tap water. Observe signs of recovery, such as gill movement and regained righting reflex, within 1 h[16].

3. Microsurgical laminectomy

NOTE: The laminectomy is performed under a stereomicroscope.

3.1. Place the animal in the prone position on the Petri dish. Wrap it in paper towels so that the tail is exposed.

NOTE: The paper towels are excellent for ensuring stability throughout the procedure.

3.2. Identify the hind limbs. Make the first incision just caudal to them.

3.2.1. With a pair of microscissors, perform a vertical incision from the keel until the bony prominence of the spinous processes are felt.

NOTE: Be very careful when grasping the keel and skin with forceps, because these easily inflict damage to the delicate skin.

3.2.2. Extend the cut laterally, so the incision traverses the entire width of the tail.

3.2.3. Grasp the spinous process with forceps to ensure the right depth.

3.2.4. Extend the vertical incisions 1 mm below the spinous process on both sides.

3.3. Place the animal on one side to perform ventral and horizontal incisions as stated below.

3.3.1. With a pair of microscissors, starting from the ventral point of the vertical incision, make a horizontal incision of approximately 15 mm for animals 10-20 g in weight. Make the incision longer for larger animals, and shorter for smaller animals.

3.3.2. Using the scissors, dissect medially through the horizontal incision until the vertebral column is felt in the midline.

3.3.3. Repeat steps 3.3, 3.3.1, and 3.3.2 on the other side of the animal.

3.4. Having dissected in the deep medial plane from both sides, dissect through the midline, thereby connecting the two horizontal incisions.

3.4.1. Move the free piece of tail and keel to one side, exposing the spinous processes (**Figure 1**).

3.4.2. Fixate the tail piece using wet paper towels.

3.5. Place the animal in the prone position again with the head facing the surgeon's non-dominant side.

3.5.1. With a pair of forceps, grasp the spinous processes just caudal to the hind limbs. Apply a gentle lift both up and towards the head of the animal.

3.5.2. Place the blades of a pair of microscissors horizontal around the process and gently cut it. The lift on the process ensures that it is now removed, exposing the spinal cord.

3.5.3. Grasp the spinous process just caudal to the one that was just removed and repeat steps 3.5.1 and 3.5.2.

NOTE: This should leave an exposed spinal cord corresponding to two vertebral levels. When performing the laminectomy, a white foamy secretion often appears. The spinal cord is easily identified by its distinctive shine, along with a vessel running along the midline.

3.5.4. Depending on the size of the animal, the exposed area may not be wide enough. Using two

pairs of forceps, grasp the laminae on both sides of the spinal cord and twist these laterally with a gentle movement.

4. Introducing a contusion type injury (Figure 2)

4.1. Keep the animal in the prone position.

4.2. Use the Petri dish to transfer the animal to the trauma unit.

4.3. Have an assistant shine a flashlight on the spinal cord.

4.4. Place the contusion trauma unit cylinder above the exposed spinal cord using the microadjusters on the unit. Aim through the cylinder.

4.5. Lower the cylinder until it is level with the laminae.

4.6. Attach the falling rod to the electromagnet. Place the desired falling height adjustment cylinder on the trauma unit.

4.7. Place the falling rod in the cylinder.

NOTE: For a blinded study, the surgeon should now leave the room without knowing if the animal will be assigned to an injury or a sham surgery group.

4.8. Turn off the electromagnet. The rod falls to the exposed spinal cord.

4.9. Use the height adjustment screw to lift the rod from the spinal cord.

4.10. Confirm the injury by looking at the spinal cord through the microscope. The injured site will appear darker, and bleeding from the midline vessel will be apparent.

5. Introducing a sharp injury

NOTE: Perform these steps after 3.5.4.

5.1. With a pair of microscissors cut the spinal cord in a perfect vertical cut.

5.2. Repeat the cut 2 mm to the caudal side of the body.

NOTE: The length of the removed piece of spinal cord can be adjusted as per the study requirement. However, a 2 mm cut will be regenerable[10].

5.3. Ensure that the cuts are complete. Upon completion, feel the blades of the scissors scraping along the ventral part of the spinal canal.

5.4. Lift the 2 mm piece of spinal cord from the spinal canal.

6. Closing the surgical wound

6.1. Return the animal to the surgical table. In a blinded study, reposition the keel so the spinal cord is not visible to the surgeon.

6.2. Keep the animal in the prone position.

6.2.1. Begin placing 10.0 nylon sutures from the most caudal part of the horizontal incision. Close the wounds in one layer.

NOTE: Do not grasp the skin too tight, because it will inflict necrosis.

6.2.2. Work towards the vertical part of the incision.

6.2.3. When reaching the angle, turn the Petri dish and suture the other horizontal incision.

6.2.4. Set sutures on the vertical incisions.

6.2.5. Do not place sutures in the uppermost part of the keel, because the skin here will not be able to hold.

7. Returning the animal to the anesthetic-free solution

7.1. Lift the Petri dish with the animal and submerge both very gently into fresh water only 5 cm deep and let the animal slide off.

NOTE: The shallow water depth ensures that the animal will not attempt to swim to the surface to breathe.

7.2. Do not change the water during the first week.

7.3. When feeding the animals, ensure that the food is placed near the animal's head.

NOTE: The purpose of these measures is to avoid as much movement as possible during the first week.

8. Postoperative ultrasound

8.1. Prior to the termination of anesthesia, use a high frequency ultrasound system to acquire images of the injury that can be used for the construction of three-dimensional images of the SCI site.

8.2. Attach the transducer to a micromanipulator preferably governed by a remote joystick.

8.3. Submerge the anesthetized animal in the prone position into a small container filled with anesthetic solution.

NOTE: Fix the animal with miniature sandbags or other equipment to avoid movement during the scanning sequence.

8.4. Align the tip of the transducer with the animal's length axis and submerge it into the benzocaine solution until it is only a few millimeters above the keel behind the hind limbs of the animal.

8.5. Identify the SCI site.

NOTE: The injury site is easily recognizable due to the missing spinous processes directly above the SCI.

8.6. Optimize the image by adjusting the ultrasound settings. Ensure that the SCI site is in the center of the image. Adjust the field of view (i.e., image depth, depth offset, and image width) to cover the SCI site and adjacent healthy tissue. Adjust the two-dimensional gain to optimize the image contrast.

8.7. By sweeping the ultrasound transducer across the SCI site with an electronically operated micromanipulator, acquire B-mode images covering the SCI site at multiple sagittal cross-sectional slice locations, with consecutive slices with an interslice interval of 50 µm. Acquire cine-images containing 500 frames with a frame rate of ~50 frames/s and a transducer frequency of 40 MHz.

NOTE: This setup requires an electronic micromanipulator governed by a remote joystick (step 8.2).

8.8. After finishing the scanning sequence return to step 7.

REPRESENTATIVE RESULTS:

The purpose of the protocol is to produce an SCI that will paralyze the motor and sensory functions caudal to the injury. Because the axolotl is regeneration-competent it restores function within weeks, allowing researchers to study CNS regeneration during a short time span.

Anesthesia was provided for 45 min to all animals, and no episodes of preterm recovery were experienced. All animals recovered within an hour and showed no signs of damage from anesthesia in the following weeks[13,16].

The laminectomy was successful in all animals. However, anatomical variation in the width of the

spinal canal called for the widening of the canal using forceps and a twist in some individuals. Furthermore, residual laminae in some individuals prevented the falling rod from reaching its target, hence making it imperative that the surgeon clean the field from the residual bone and prominences.

Closing the incisions was associated with some difficulties, especially during the piloting phase of the study. Sutures in the top part of the keel would not hold and resulted in insufficient closures. The closure of one animal in the study did not hold, resulting in the keel being torn, subsequent infection, and death. This stresses the need for careful suturing along the entire incisions.

The initial mechanical injuries were obvious during the procedure. During the model development, injured and sham animals were stained with hematoxylin and eosin to validate the injury. Representative results of each group are shown in **Figure 3A1**,**A2** and **Figure 3C1**,**C2**. Regeneration was confirmed by histological sections preparations made after nine weeks (**Figure 3B1**,**B2** and **Figure 3D1**,**D2**), which showed a reestablished spinal cord connection in the SCI animals.

Injury and regeneration can be followed by examining neurological function. Stimulating the tail with a light touch and pinching from forceps will reveal whether tactile and nociceptive sensory functions have been lost and potentially reestablished. A neurological score was defined based on the reaction of the animal: 0 point = no response, 1 point = local tail movement, 2 points = truncal movement, 3 points = coordinated movement of limbs and/or head alongside with truncal movement, 4 points = animals with immediate coordinated fast movement. In six SCI animals versus five sham animals the loss of neurological function three weeks post injury was found, and a gradual restoration within nine weeks (**Figure 4** and **Supplementary Video 1**).

Ultrasonographic images of the injured spinal cord can be obtained using the above protocol. Visualizing the SCI site was possible due to the obvious lack of bony spinous processes (**Figure 5**). Furthermore, using the B-mode the dorsal artery of the uninjured spinal cord could be visualized, yielding a marker of vessel integrity.

It is possible to test the animals immediately upon reawakening. However, some animals expressed local small amplitude, repetitive, and rhythmic tail movement upon stimulation comparable to the clonus phenomena observed in human SCI. These movements might represent clonus or a lack of central reflex suppression and could potentially cause more damage to the newly injured spinal cord. Therefore, testing the animals is not recommend before one-week post injury.

From simple qualitative observation of the animals, it will be evident that the tail is paralyzed, and swimming is significantly inhibited, making the animals completely dependent on moving their limbs. These observations will also validate the success of the protocol.

High-field MRI scans (9.4 T) were performed immediately after injury to visualize the injury in vivo (**Figure 6**). However, the scans were generally low in signal-to-noise ratio compared to those

of non-operated animals, likely due to bleeding and hemosiderin. Hence, it was concluded that MRI was a suboptimal method to validate the injury and success of the protocol.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic drawing of the microsurgical laminectomy.

Figure 2: Schematic drawing of the contusion trauma mechanism. (**A**) The entire setup, showing the falling rod above the animal. (**B**) The disassembled mechanism, showing how the rod is disconnected from the electromagnet. (**C**) The falling rod is connected to the electromagnet. The falling height adjustment cylinder is installed, and the electromagnet and rod loaded into the cylinder. Height adjustment of the entire system is controlled by an adjusting wheel. (**D**) Turning off the electromagnet will cause the rod to fall without the operator touching the system. Figure was originally published by Thygesen et al.[13].

Figure 3: Histological sections hematoxylin and eosin stained immediately and nine weeks post injury. (**A1**) SCI animal immediately after injury. (**B1**) SCI animal at nine weeks. (**C1**) Sham surgery animal immediately after injury. (**D1**) Sham animal at nine weeks. Red square = marks the injury of the SCI animals, and the laminectomy of the sham animal. **Figures A2, B2, C2** are magnifications of these areas at 5x. Blue arrow = uninjured spinal cord. This figure was originally published by Thygesen et al.[13].

Figure 4: Graph of response to tactile stimuli. The response of the SCI groups is lower after three weeks, compared to the sham group. WPI = weeks post injury, Black line = SCI, Grey color = sham. Sham n = 5, SCI n = 6. Figure was originally published by Thygesen et al.[13].

Figure 5: Ultrasonographic image showing the spinal cord in a sagittal section. Yellow lines mark the spinal cord, yellow circle the injury site, and white arrows mark the vertebrae.

Figure 6: MRI scans at different time points post injury or sham surgery. CSF surrounding the spinal cord is lacking, especially at three WPI for the SCI animal, indicating swelling of the spinal cord. Darkening of the spinal cord indicates edema as well. Notice how these changes disappear as regeneration progresses. Yellow arrow = the area of laminectomy. Figure was originally published by Thygesen et al.[13].

Supplemental Video 1: Video showing the neurological function after tactile stimuli and later a nociceptive stimulus. First a healthy control animal, and then an animal suffering from SCI.

DISCUSSION:

Because risk of injury to the spinal cord is significant, the critical steps of the protocol are removing the spinous processes and widening of the bony access to the spinal canal if needed. As mentioned in the protocol, removing the most cranial process first is highly recommended. This will mean that the more caudal processes protect the spinal cord from being hit by the scissors. It is recommended to ensure enough surgical access, meaning to not make too small a primary incision. Also, when grasping anything with forceps, the direction of the pull applied must

always be considered. Applying a gentle pull away from the spinal cord will protect it in the event of the grasp failing and a slip of the instrument.

The surgical procedure in the axolotl is not different from other animals. However, certain important differences do exist, primarily attributable to the tissue composition and size of the animal. The axolotl keel skin is very fragile, and paradoxically does not heal well upon small damages inflicted during incision. Caution should be taken, especially upon the primary incisions, because damage will substantially complicate the suturing. The bones of very young axolotls are very soft. This means that often basic anatomical forceps may suffice in bone removal. This presents another element of caution, because pinching the spinous processes could inflict substantial damage. The subcutaneous and muscle fascia layers are not available for suturing, due to their fragile tissue compositions. It is imperative to ensure a calm postoperative week. The animals may not rest sufficiently after the operation. Hence, they may inflict secondary damage to their spinal cord postoperatively. Their small anatomy does not allow for neither internal nor spline fixation.

Weight and falling height of the falling rod system is crucial to inflicting a contusion injury. During extensive piloting for an earlier study, the rod weight and falling height needed was found to be 25 g and 3 cm[13]. This was enough to induce paralysis in 12 g axolotls without cutting or disintegrating the spinal cord. Added weight or falling height might be needed in bigger animals. Furthermore, the diameter of the falling rod might need to be bigger in the case of bigger animals and shorter for smaller animals.

The model has some limitations. Because axolotls are not used for learned behavior studies, one cannot test complex neurological functions. The injury was introduced caudal to the limbs, sparing the hind limbs and bowel and bladder from being paralyzed. The reason for this was ethical, to reduce the impact on the animal to a minimum. However, it does limit the opportunity to study the effects on limb movements, which may be easier to describe and categorize. A large part of the SCI-associated morbidity stems from the loss of control of bowel and bladder. This model does not allow for future research in these fields. Inflicting damage rostral to the hind limbs would be possible, but it was not attempted.

Studying SCI in a regenerative model such as the axolotl allows for a different approach in SCI research. Because the animal model can regenerate, elimination studies will be able to reveal critical factors of regeneration. Conventional studies on SCI are performed in non-regenerative models, meaning that one will need to intervene on all critical factors to induce a regenerative response.

This model and protocol are in concordance with Krogh's principle stating that: "For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied"[17]. Mammalian regeneration is inhibited by multiple factors. Inhibiting these in a mammalian model usually does not induce any effects. However, increasing levels of inhibitors in the axolotl should eliminate regeneration, and thereby reveal whether that inhibitor is critical or not[10].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1 vector version

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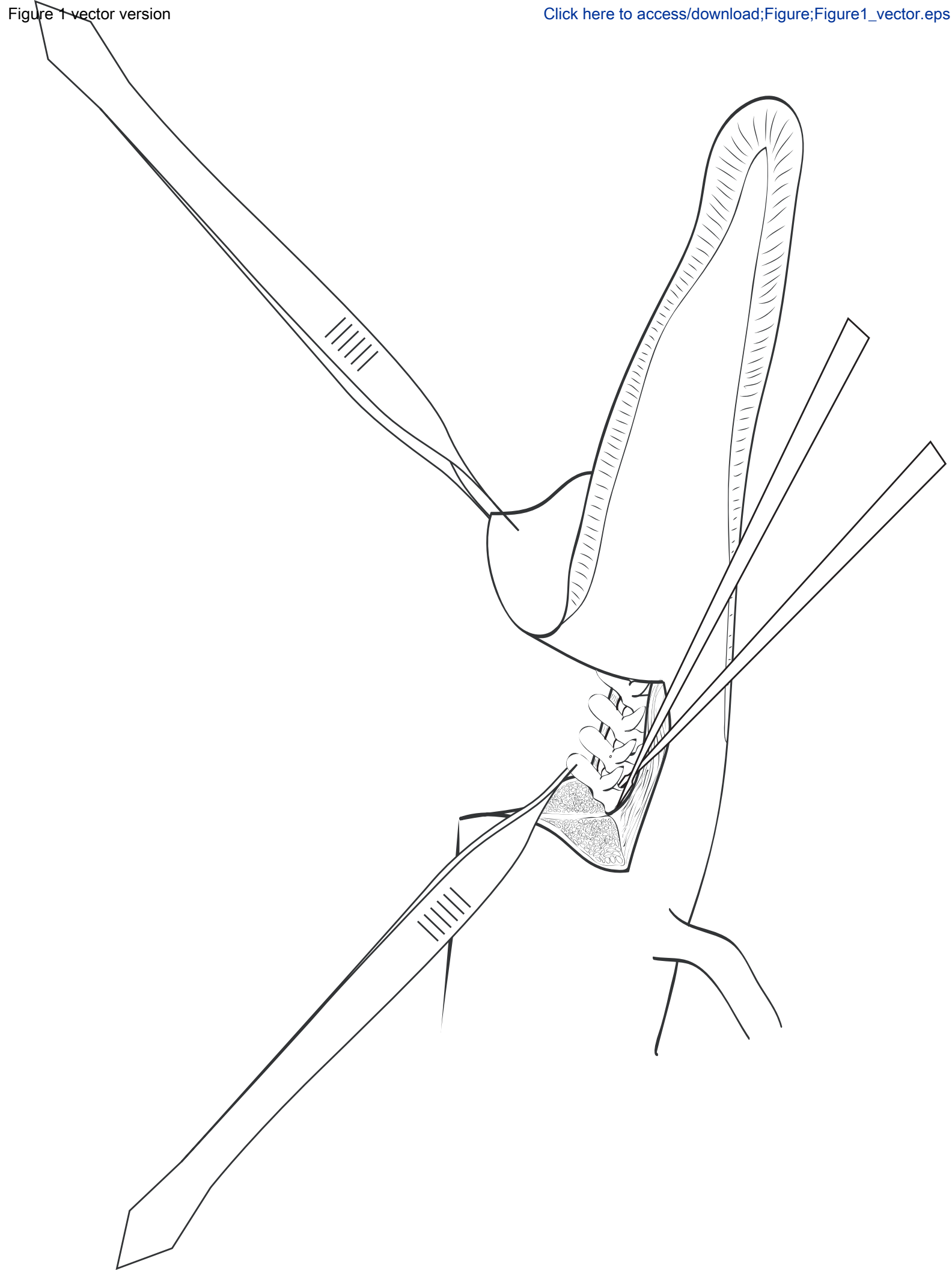

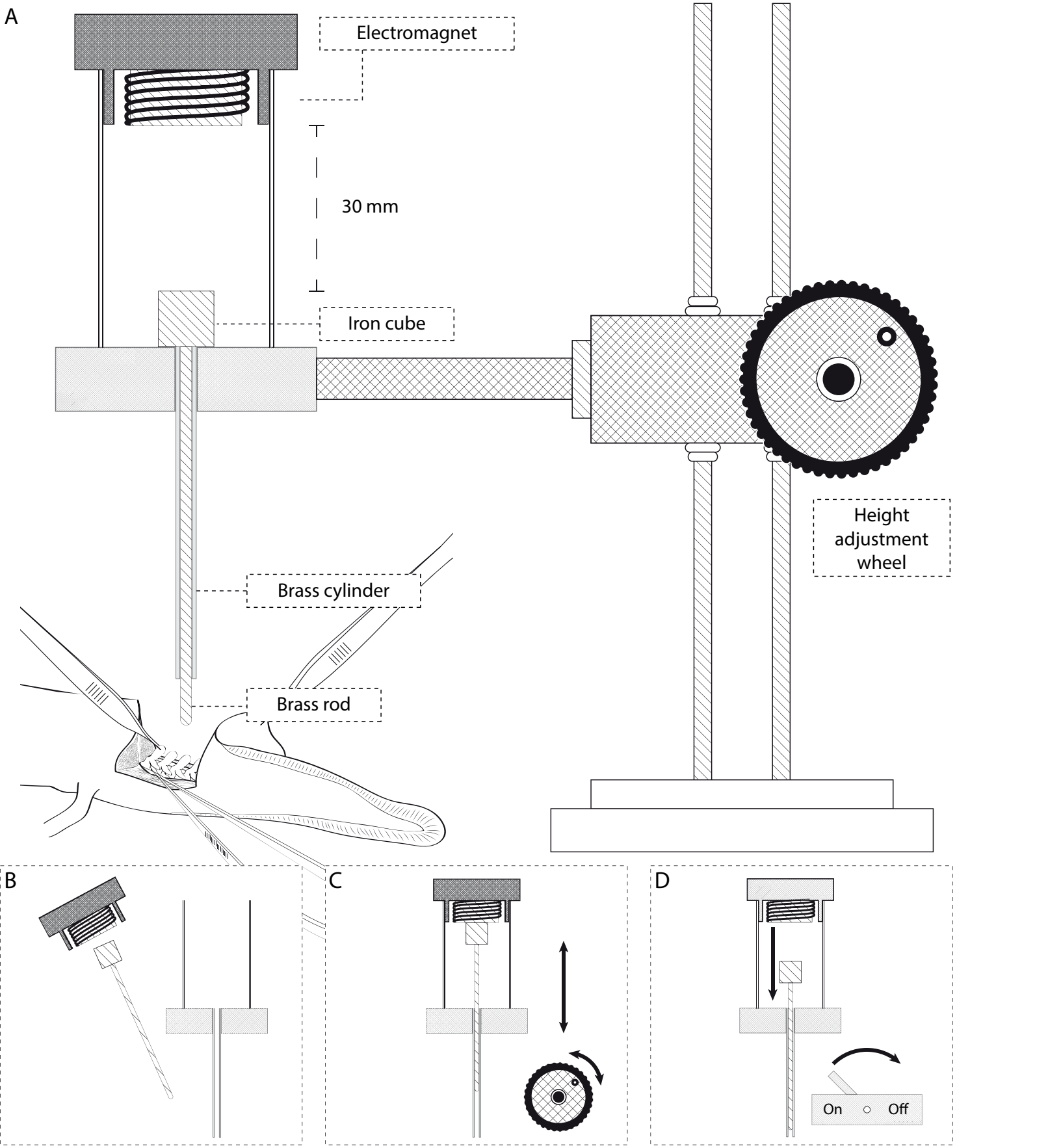

Figure 3

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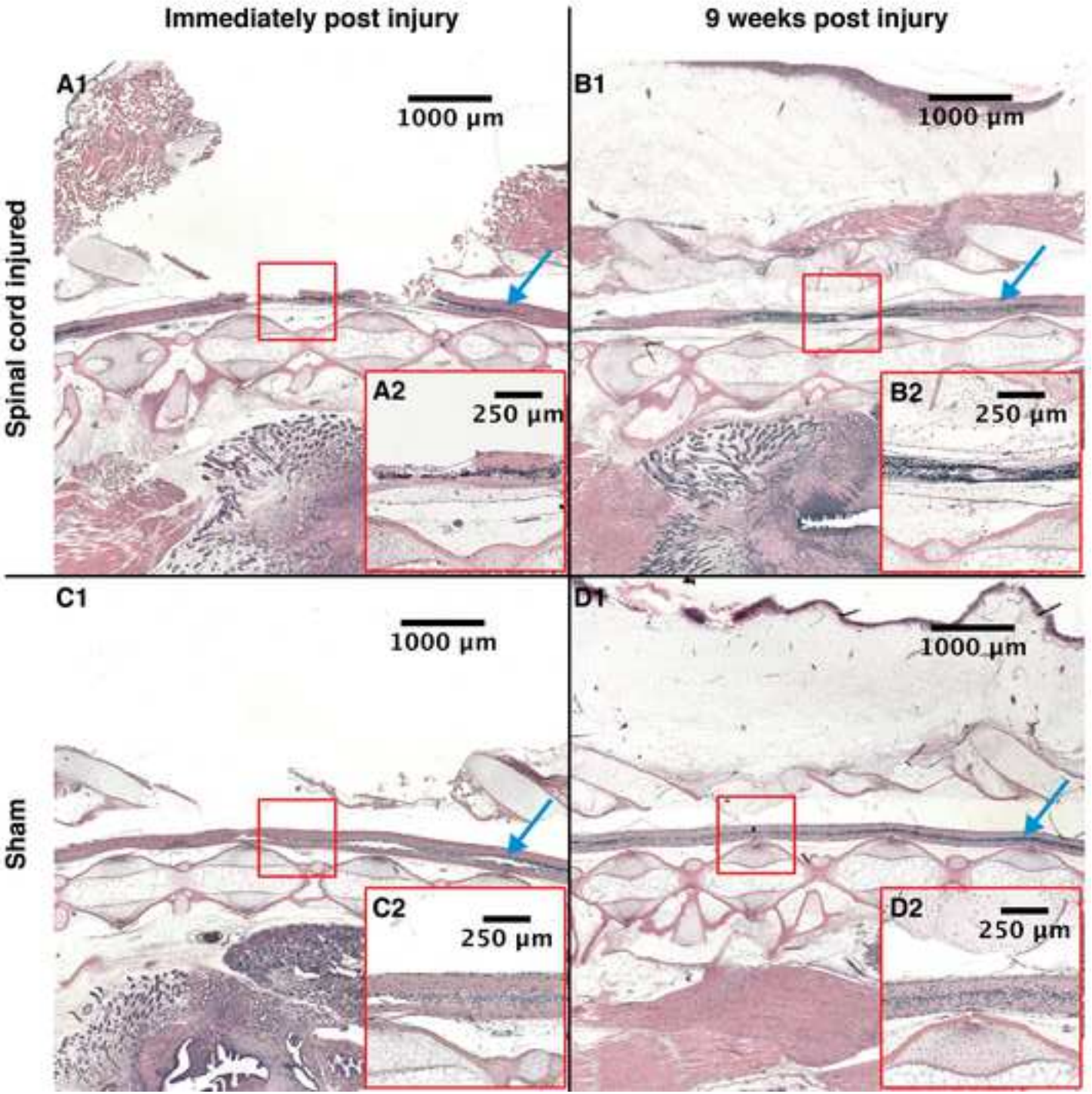

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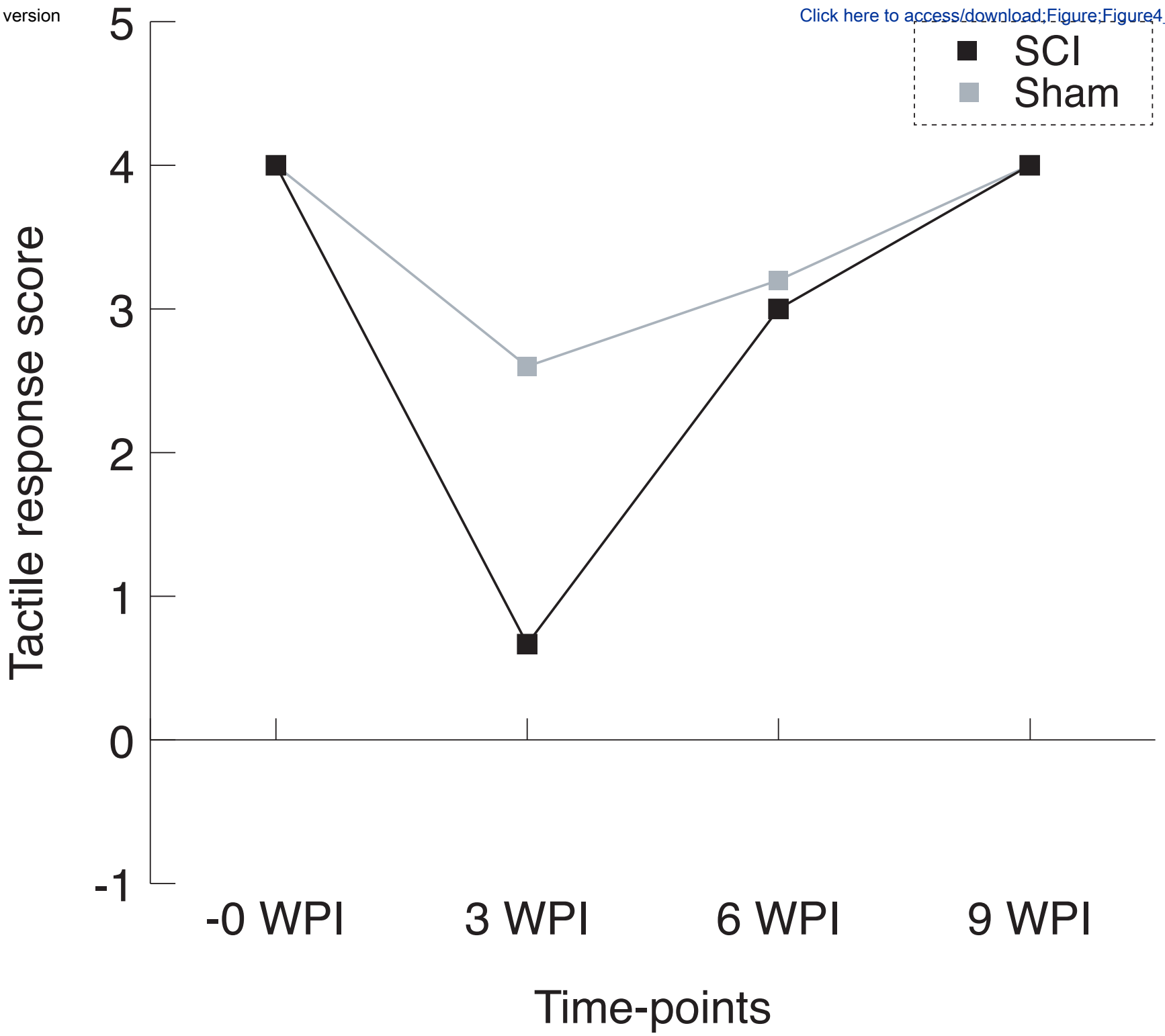

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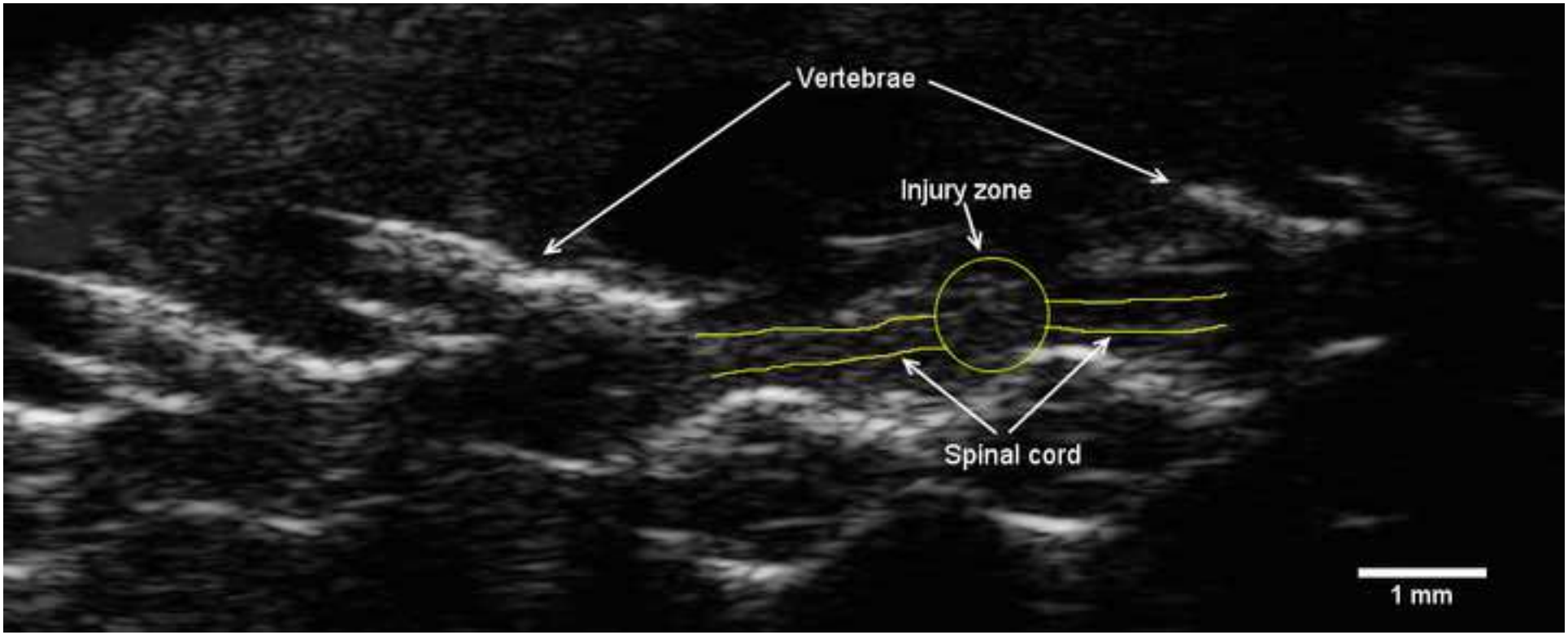
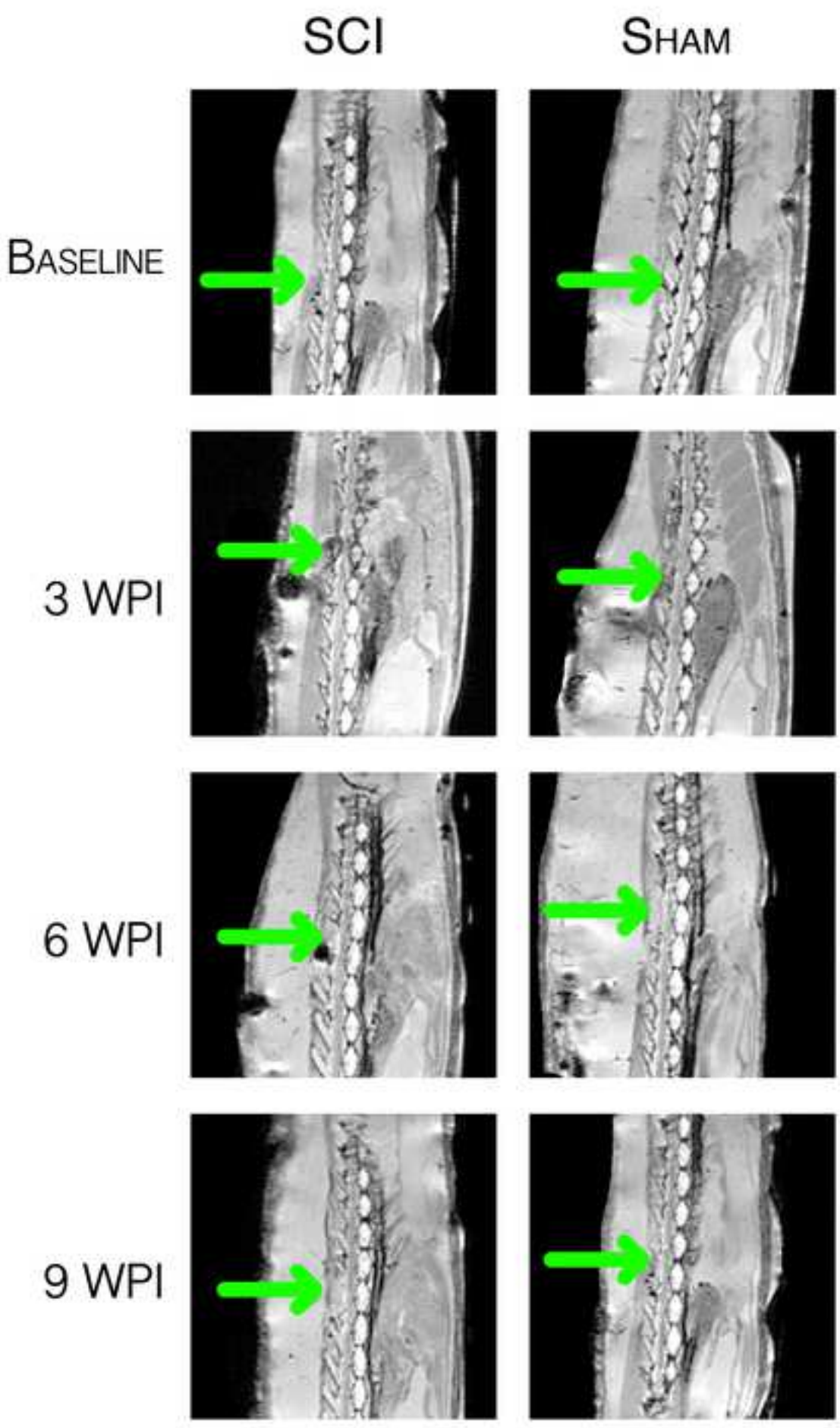


Figure 6



Supplementary Video 1

Supplementary Video 2_for production

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Video or Animated Figure

480 - SD 480p.mov

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
25 g custom falling rod	custom home made	
30 mm PVC pipe	custom home made	
Acetone	Sigma-Aldrich	67-64-1
Axolotl (Ambystoma mexicanum)	Exoterra GmbH	N/A
Benzocain	Sigma-Aldrich	94-09-7
electromaget	custom home made	
Excel 2010	Microsoft	N/A
ImageJ	National Institutes of Health	
kimwipes		
microsurgical instruments	N/A	N/A
MS550s	Fujifilm, Visualsonics	MS550s
MS700	Fujifilm, Visualsonics	MS700
Petri dish	any maker	
Soft cloth	N/A	N/A
Stereo microscope		
Vevo 2100	Fujifilm, Visualsonics	Vevo 2100

Comments/Description

Propanone
12-22 cm and 10 g - 80 g, All strains (wildtype, melanoid, white, albino, transgenic white with GFP)

ethyl 4-aminobenzoate

Excel 2010 or newer
ImageJ 1.5e or newer. Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 19

Forceps and scissors
40 MHz center frequency, transducer
50 MHz center frequency, transducer

Any piece of soft cloth measuring appromixately 70 x 55 cm^2 e.g. a dish towel

High frequency ultrasound system

)97-2016.

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Title of Article: Contusion spinal cord injury via a microsurgical laminectomy in the regenerative axolotl

Author(s): Mathias Møller Thygesen, Fredrik Guldbæk-Svensson, Mikkel Mylius Rasmussen, Henrik Lauridsen

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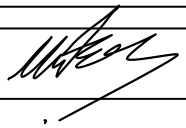
Signature:	
Date:	30. of May 2019

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Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please provide at least 6 keywords or phrases.

Line 23: "Ultrasonography" added

3. Please rephrase the Long Abstract to more clearly state the goal of the protocol

Line 30-34: "The purpose of this study was to establish a reproducible regenerative spinal cord injury model in the axolotl. Enabling the future research on spinal cord injury regeneration."

4. Please revise the Introduction to include all of the following with citations:

a) A clear statement of the overall goal of this method

Line 59-61: Rephrased: "The overall goal of this study was to establish a controlled and reproducible microsurgical method for inflicting blunt and sharp spinal cord injuries (SCI) to the axolotl (Ambystoma mexicanum) spinal cord, in turn yielding a regenerative spinal cord injury model."

b) The rationale behind the development and/or use of this technique

We believe that this is answered in line 75-80: "Whereas complete appendage amputation in the axolotl results in full regeneration, some non-amputation based regenerative phenomena are dependent on the critical size defect (CSD)14-15, meaning that injuries that exceed a critical threshold are not regenerated. Therefore, we found a need forthis study investigating investigated whether a 2 mm blunt trauma would exceed the CSD limit, with the purpose of developing a regenerative model with a higher clinical translational value."

c) The advantages over alternative techniques with applicable references to previous studies

We believe that this is answered in line 73-80: "Most SCI studies in the axolotl are performed as either amputation of the entire tail or ablation of a larger part of the spinal cord9-12. Recently a new study was published on blunt injuries13 which mimics the clinical situation better. Whereas complete appendage amputation in the axolotl results in full regeneration, some non-amputation based regenerative phenomena are dependent on the critical size defect (CSD)14-15, meaning that injuries that exceed a critical threshold are not regenerated. Therefore, we found a need forthis study investigating investigated whether a 2 mm blunt trauma would exceed the CSD limit, with the purpose of developing a regenerative

model with a higher clinical translational value."

d) A description of the context of the technique in the wider body of literature

We believe that this is answered in line 73-80: "Most SCI studies in the axolotl are performed as either amputation of the entire tail or ablation of a larger part of the spinal cord9-12. Recently a new study was published on blunt injuries13 which mimics the clinical situation better. Whereas complete appendage amputation in the axolotl results in full regeneration, some non-amputation based regenerative phenomena are dependent on the critical size defect (CSD)14-15, meaning that injuries that exceed a critical threshold are not regenerated. Therefore, we found a need forthis study investigating investigated whether a 2 mm blunt trauma would exceed the CSD limit, with the purpose of developing a regenerative model with a higher clinical translational value."

e) Information to help readers to determine whether the method is appropriate for their application

We believe that this is answered in line 82-85: "This method is relevant for readers working on spinal cord regeneration in small animal models, especially in the axolotl. Furthermore, it may be of more general interest, since it exhibits a way of utilizing standard laboratory equipment to develop a blunt trauma mechanism which is suitable for use in small animals in general."

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Kimwipes **(Changed to paper towels)**, **Vevo2100, 262 Fujifilm VisualSonics, Toronto, Canada, a MS550D transducer (removed)**

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Line 87-90: "ETHICS STATEMENT:
All applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this study. The study was conducted under the approval id: 2015-15-0201-0061 by the Danish Animal Experiment Inspectorate." Added.

7. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Line numbers here refer to the first submitted version of the manuscript, since many of the changes include removals.

Line 26: “Here protocols for surgically inflicting controlled blunt and sharp spinal cord injuries to a regenerative axolotl (*Ambystoma mexicanum*) are presented.”

Line 41: “We found that” removed

Line 44: “We found” removed

Line 56: “We found” changed to “it was found”.

Line 74: Rephrased to “Therefore, this study investigated whether a 2 mm blunt trauma would exceed the CSD limit, with the purpose of developing a regenerative model with a higher clinical translational value.”

Line 125: Changed to “Observe reduced movements and increasing loss of righting reflex within 20 minutes.

Line 198: “Using the scissors dissect in the depth of the horizontal incision, until the vertebral column is felt.”

Line 310: Rephrased to passive tense.

Line 317: Rephrased to passive tense.

Line 332: “we found” removed.

Line 335: “we do not recommend” removed

Line 341: Rephrased to passive tense.

Line 342: “hence it was concluded that MRI was a suboptimal method to validate the injury and hence success of the protocol.”

Line 408: “you apply” changed to applied.

8. Please include a single line space between each step, substep and note in the protocol section.

Line space added before all notes.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Step 1.1.1: Reprashed to “Use High quality non-chemically treated tap water. If unavailable, use 40% Holtfreter’s solution.”

Step 2.2: Rephrased to “Observe reduced movements and increasing loss of righting reflex within 20 minutes.”

Step 2.3: Rephrased to: “Observe signs of general anesthesia within 30-45 minutes. Gill movements will be lacking and the animal will not respond to neither tactile nor painful stimuli (gentle pinching of toe web)”

Step 2.4: Rephrased to: “To maintain anesthesia, wrap the animals in paper towels wetted in anesthetic solution. Wet these regularly with solution during the surgical procedure, to ensure that the skin and gills are kept moist.”

Step 2.5: Rephrased to: “Recover the animal after surgery by placing it in a container containing fresh tap water. Observe signs of recovery, such as gill movement and regained righting reflex, within 1 h.”

Step 3.2: Rephrased to “Identify the hind limbs, make the first incision just caudal to these”

Step 3.2.4: Rephrased to “The vertical incision is now almost finished. However, vertically extend it 1 mm below the spinous process on both sides.”

Step 3.3.2: Rephrased to “Using the scissors dissect in the depth of the horizontal incision, until the vertebral column is felt.”.

Step 3.4: Rephrased to “Having dissected in the deep from both sides, dissect through the midline, hereby connecting the two horizontal incisions.”

Step 3.4.1: Rephrased to "Move the free piece of tail and keel to one side, exposing the spinous processes (Fig. 1)."

Step 3.5.1: Rephrased to "With a pair of forceps, grasp the spinous process just caudal to the hind limbs. Apply a gentle lift both up and towards the head of the animal."

Step 3.5.2: Rephrased to "Place the blades of a pair of micro scissors horizontal around the process and gently cut the process. The lift on the process ensures that it is now removed, exposing the spinal cord."

Line 272: changed to note instead of step.

Line 275: 4.1.8 and 4.1.9 steps merged.

Step 6.1: Rephrased to: "6.1)Return the animal to the surgical table. In a blinded study, reposition the keel so the spinal cord is not visible to the surgeon. "

Steps 6.3 and 6.4 merged to one note.

Step 8.1: Rephrased to "Prior to termination of anesthesia, use a high frequency ultrasound system to acquire images of the injury, which can be used to construct 3D images of the SCI site."

Step 8.7: Rephrased to: "Acquire B-mode images covering the SCI site at multiple sagittal cross-sectional slice locations, with consecutive slices with an interslice interval of 50 µm. Acquire cine-images containing 500 frames with a frame rate of ~50 frames/s and a transducer frequency of 40 MHz."

10. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

See the corrections above.

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

See the above corrections, no steps are now more than 3 lines.

12. Please remove the redundancy from the protocol and make the steps crisps clearing describing how the protocol is performed in a stepwise manner.

Step 1.2 removed

Note removed: "Note: Anesthesia can be maintained for up to 7 hours this way with no observable adverse effects to animal welfare after recovery."

Step 3.2.4 shortened: "3.2.4) Extend the vertical incisions 1 mm below the spinous process on both sides."

Note removed: "Note: The surgeon must be able to use scissors with both hands."

Note removed: "Note: The bone is soft enough to be twisted. The forceps can also be used to remove small bony prominences if needed.

Note removed: "Note: Do no try to cut the laminae, the epidural space is simply too small to introduce scissors here."

13. Please ensure you answer the "how" question, i.e., how is the step performed?

14. 2.1: Please do not highlight anesthesia steps.

Highlight removed

15. Line 122-123: Citation if any?

"2.5) Recover the animal after surgery by placing it in a container containing fresh tap water. Observe signs of recovery, such as gill movement and regained righting reflex, within 1 h." Citation added.

16. 8.6: how is this done?

17. 8.7: Please include all the button clicks, knob turns etc. performed in the instrument and associated softwares.

Since we do not find this part to be the main focus of the protocol, we have added a short description of how it is performed. We would like to refer to an earlier JoVe article we have published on ultrasonography and 3d reconstruction if you find it appropriate?

18. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that

identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The highlighted text should be approximately 2.5 pages long.

19. Please ensure each section of the protocol has a representative result associated with it.

2. Anesthesia:

Added to the results: "Anesthesia was obtained within 45 minutes for all animals, and no episodes of preterm recovery was experienced. All animals recovered within an hour and showed no signs of damage from anesthesia in the following weeks."

3. Microsurgical laminectomy:

Added to the results: "The laminectomy was successful in all animals. However, anatomical variation in the width of the spinal canal called for widening of the canal using forceps and a twist in some individuals. Furthermore residual laminae would in some individuals prevent the falling rod from reaching its target, hence making it imperative that the surgeons clean the field from residual bone and prominences."

4. introducing the contusion injury and 5. Introducing the sharp injury:

We find that the existing section is associated with the protocol sections 4 and 5:

"The initial mechanical injuries were obvious during the procedure, however during model development injured and sham animals were stained with hematoxylin and eosin to validate the injury. Representative results of each group are shown in Fig. 3A1-A2 and Fig. 3C1-C2. Regeneration was confirmed by these histological sections made after 9 weeks (Fig. 3B1-B2 and Fig. 3D1-D2), which showed a reestablished spinal cord connection in the SCI animals.

Injury and regeneration can be followed by examining neurological function. Stimulating the tail with a light touch and pinching from forceps will reveal whether tactile and nociceptive sensory functions have been lost and potentially reestablished. A neurological score was defined based on the reaction of the animal:

0 point: no response. 1 point: local tail movement. 2 points: truncal movement. 3 points: coordinated movement of limbs and/or head alongside with truncal movement. 4 points: animals with immediate coordinated fast movement. In 6 SCI animals vs 5 sham animals a loss of neurological function 3 weeks post injury was found, and a gradual restoration within 9 weeks (Fig. 4).

It is possible to test the animals immediately upon reawakening. However, some animals expressed local tail movement upon stimulation in a somewhat clonus like manner. These movements might represent clonus or a lack of central reflex suppression, and could

potentially cause more damage to the newly injured spinal cord. Therefore, testing the animals is not recommend before one week.

From simple qualitative observation of the animals, it will be evident that the tail is paralyzed, and swimming is significantly inhibited, making the animals completely dependent on moving their limbs. These observations will also validate the success of the protocol.

High-field (9.4 T) MRI scans were performed immediately after injury in an attempt to visualize the injury in vivo. However, the scans were generally low in signal to noise ratio compared to those of non-operated animals, likely due to bleeding and hemosiderin, hence it was concluded that MRI was a suboptimal method to validate the injury and hence success of the protocol."

6. Closing the surgical wound:

Closing the incisions was associated with some difficulties, especially during the piloting phase of the study. Sutures in the top part of the keel would not hold, and resulted in insufficient closures. The closure of one animal in the actual study did not hold, resulting in initially the keel being torn subsequent infection, and death. This stresses the need for careful suturing along the entire incisions.

7. Returning the animal to the anesthetic free solution:

See the above additions under anaesthesia.

8. Postoperative ultrasound:

Representative postoperative ultrasound figure added.

20. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

Figure 1 and 2 referenced in the protocol.
Figure 3 and 4 referenced in results.

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Approval letter uploaded.

22. As we are a methods journal, please revise the Discussion to explicitly cover the following in

detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Answer: We have generally rearranged and revised the discussion to follow the structure and content proposed. Generally we do not find "Any modifications and troubleshooting of the technique" to very relevant for this protocol.

23. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

Check

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Sorted

25. Please fill out item 1 and item 2 in the attached ALA and sign the ALA as well. Please upload the ALA to your editorial manager account

Sorted

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is a methods paper describing the generation of a robust assay for spinal chord injury using the axolotl model. The authors describe their surgical procedures to reveal a section of the spinal chord, and the use of a trauma device to generate reproducible injuries. Overall, this method will be useful to the community, and the procedural steps provide a lot of detail for others to reproduce the technique. While I have no major concerns about this paper, there are a number of minor concerns that I have listed below that I feel will improve the paper.

Major Concerns:
None

Minor Concerns:

Abstract: Lacking the description of big picture point of it all. It would be good to explain that axolotl can regenerate their spinal chord, but a reproducible assay to study this process has not been established. I would explain why type of spinal injury (if it were to occur on humans) the injury in the current paper is tying to mimic. I also recommend also explaining why a trauma device is needed -what makes it better than surgically generating a contusion.

Answer: We have rephrased the abstract introduction in line 30-33:
"The purpose of this study was to establish a standardized and reproducible regenerative blunt spinal cord injury model in the axolotl. Most clinical spinal cord injuries occur as high energy blunt traumas, however most studies in the axolotl spinal cord has been conducted with sharp traumas. Hence this study aim to produce a more clinically relevant regenerative model."

Intro: Short but to the point.

Protocol: For some reason there was a lot of yellow highlighting in the version the authors uploaded -maybe they accidentally uploaded a previous version of the manuscript?

Answer: These are highlights of the suggestions for filming.

Line 88: units of tap water volume not present. Typo of "tap" as is "ltap"….

Answer: unit L added to line 111, and misspelling corrected.

Section 2: What sized axolotls are you performing the surgery on. The timing of anesthesia will likely be greatly different depending on the size of the animal.

Added to the materials list.

Line 108: typo

Answer: fixed in step 2.3. "respond to"

It is very surprising that the authors can keep the animals unconscious for up to 7 hours with just wet kim wipes -my lab also uses MS222, and we havent gotten close to this time. DO the authors put the animals on ice to slow the processing of MS222 in the anesthetized animals?

Answer: We do not have published data on this, and therefor cannot reference it. However doing piloting of our MRI scans and during other studies (heart regeneration) we have had success with long term anaesthesia of about 7 hours. During these experiment we did continuously wet the wipes with anesthetics. We have removed the note from the manuscript to avoid any confusion.

The authors describe the measurements of the different incisions, however where this is depends greatly on the size of the animal the surgery is performed on. It would be helpful if the authors provide more information on the anatomical features that they used to guide them.

Anwer: We agree with this point. The 15 mm were chosen from experience, that it would suffice to expose the spinal column anough for a two level laminectomy. We have added the following to step 3.3.1: "With a pair of micro scissors, starting from the vental point of the vertical incision, make a horizontal incision of approximately 15 mm, for animals of a size of 10-20 g. Make the incision longer for larger animals and vice versa."

It would also be helpful if they explained why they make the incision location where they do. Is there a specific reason for this location?

Answer: line 548-550: "skills, one cannot test complex neurological functions. The injury was introduced caudal to the limbs, sparing the hind limbs and bowel and bladder from being paralyzed. The reason for this was ethical, to reduce the impact on the animal to a minimum."

For example figure 1 could be added upon to provide a more stepwise description of what needs to happen in each step.

Answer: Since the final product will be a video exhibiting that exactly, we do not find the need for adding to the figure.

3.54) It is unclear what the authors mean by "two levels" of spinal chord. DO they mean to say that the surgery would expose the spinal chord running through two vertebral units? an image of this in a figure would be helpful.

Answer: in line 277, which has been changed to a note:

"Note: This should leave an exposed spinal cord corresponding to two vertebral levels."

Section 4. Provide more details on the trauma device. What is the weight and size of the cylinder. What force does the impact impose on the tissues. These are important details for others to be able to reproduce this protocol.

4.1.7) How is the desired falling height determined? What is the specific objective beyond the overall goal of injuring the spinal chord

Added to the discussion: "Weight and falling height of the falling rod system is crucial to inflicting a contusion injury. During extensive piloting for an earlier study, the rod weight and falling height needed was found to be 25 g and 3 cm13. This was found to be sufficient to induce paralyzes in 12 g axolotls, without cutting of disintegrating the spinal cord. Added weight or falling height might be needed in bigger animals. Furthermore the diameter of the falling rod might need to be bigger, in the case of bigger animals, vice versa.

5.1.2) it is likely that this length will vary depending on the size of the animal. Please provide details on the size of the animal that this surgery is being performed on. And, if the size of the animal doesn't matter, it should be mentioned.

Answer: added to step 5.1.2: "5.1.2)Repeat the cut 2 mm caudal. The length of the removed piece of spinal cord can be adjusted to whatever, the objective of the given study might be. 2 mm will however be regenerable."

Line 237: typo

Answer: fixed in note related to step 6.2.1. "too"

Line 310: Explain more about what you mean by "clonus like" behavior

Answer: in line 504-505: "It is possible to test the animals immediately upon reawakening. However, some animals expressed local small amplitude, repetitive and rhythmic tail movement upon stimulation comparable to the clonus phenomena observed in human SCI."

Figure 3 legend: "C1: Sham animal at immediately" -"at" should be removed…

Removed

Page 21: looks like text from the previous page ran into this page.

Assuming that we are talking about page 12, it should now be fixed.

Reviewer #2:

Manuscript Summary:

Axolotl is a useful model for studying spinal cord injury, due to its ability to achieve complete regeneration. The authors describe here a method to conduct contusion injury to the axolotl spinal cord, which is more clinically relevant to humans, and successfully results in a loss of tail

functions.

Major Concerns:
1) Abstract: The authors should provide some background information for this method, for example why the axolotl is used to study spinal cord regeneration and why do we need a contusion injury model, to tell readers why the method is relevant. This should be briefly mentioned in the abstract, and expanded upon in the introduction.
Meanwhile, only the main steps of the steps of the protocol should be mentioned in the abstract, and discussion of tricky steps could be left out.

Generally the abstract has been rephrased to accommodate this.

We believe that this paragraph of the introduction argues why a contusion model is relevant:
Most SCI studies in the axolotl are performed as either amputation of the entire tail or ablation of a larger part of the spinal cord9-12. Recently a new study was published on blunt injuries13 which mimics the clinical situation better. Whereas complete appendage amputation in the axolotl results in full regeneration, some non-amputation based regenerative phenomena are dependent on the critical size defect (CSD)14-15, meaning that injuries that exceed a critical threshold are not regenerated. Therefore, this study investigated whether a 2 mm blunt trauma would exceed the CSD limit, with the purpose of developing a regenerative model with a higher clinical translational value.

I found that critical steps were mandatory in the abstract, so for now we will leave this in the abstract.
"The critical steps of the protocol were removing the spinous processes without inflicting damage to the spinal cord. This step required training to ensure a safe procedure. Furthermore it was found that wound closure was highly dependent on not inflicting unnecessary damage to the skin during incision. "

Added to the abstract:
Axolotls are widely used as positive models in regenerative studies, due to their impressive ability regenerate almost any tissue, and have been used extensively in spinal cord injury studies.

2) Please indicate clearly axolotls of what size are we talking about in this protocol, as they can range from 1.5 to 20cm or more, which clearly affects whether and how this protocol could be

applied. The authors should also comment on how certain parameters such as incision lengths would change when using animals of different sizes than the one indicated.

Answer: in line 95 added: "Animals were Mexican axolotls (Ambystoma mexicanum) (mean body mass ± STD: 12.12 g ± 1.25 g)."
Incisions lengths have been commented upon above.

3) For the surgery instructions, please consider using terms such as dorsal, ventral, lateral, medial etc. for more clear explanations. For example on line 142: "from the ventral point of the vertical incision, cut caudally for 15 mm"

Answer: Step 3.3.1 rephrased: "With a pair of micro scissors, starting from the vental point of the vertical incision, make a horizontal incision of approximately 15 mm, for animals of a size of 10-20 g. Make the incision longer for larger animals and vice versa."
Step 3.4 "medially" added.

Minor Concerns:

58-59 Outcome of an injury is dependent on the location and extent of injury

Answer: added to line 69: "SCI is a severe condition which, depending on the level and extent, inflicts neurological disability to the extremities along with impaired bladder and bowel control[1-3]."

61-62 This is incorrect, as even human spinal cords undergo spontaneous sprouting and circuit rewiring in the uninjured parts, recovering certain neurological functions, dependent on how much spinal cord tissue is spared.

Answer: we agree, and have changed function to tissue in line 73. Tissue is not regenerated

144 Not very clear what this means

Answer: step 3.3.2. has been changed to: "Using the scissors dissect medially through the horizontal incision, until the vertebral column is felt in the midline."

188 A lamp would be sufficient?

Answer: yes, but difficult to handle. We recommend a flashlight.

201, 227 In my opinion, blinding could be done simpler by someone else randomizing the animal labels afterwards

Answer: We agree, and we do this as well, and furthermore blind all data after being digitalized. However, some animals will have easily recognizable traits. So to ensure that investigators cannot bias the results in any way, the surgeon leaves the room as well.

215 There is no step 3.5.6

Answer: in line 365 changed to 3.5.4

221 How to ensure the cuts are complete?

Answer: added to step 5.1.3: "5.1.3)Ensure that the cuts are complete. You will feel the blades of the scissors scraping along the ventral part of the spinal canal".

343 Typo: compared to sham group

Fixed in legend to figure 4.

376 Please explain if performing this protocol rostral to the hind limbs is an option

Answer: line 638: "Inflicting damage rostral to the hind limbs would be possible, but we did not attempt this."

Video: The videos are not referred to in the manuscript. In the second video, it would be better to use a sham operated animal for comparison

Answer: Added to line 522: "and supplementary video: Neurological"
We can produce a new supplementary video if the editor wishes it. However, we will need some time to do some filming in that case, and cannot make it before the deadline.

Reviewer #3:

Manuscript Summary:

This is a goo description of a SCI Protocol for axolotl spinal cord regeneration studies. No major concerns were found.

Minor Concerns:

1.1.1) It is easier to understand if the composition of the Holtfleter's solution is described.

Answer: This solution can be found if needed, we do not find it necessary to implement in the protocol.

1.1.2） 1_ltap -> 1l_tap

Answer: addressed above.

1.2.2） Is sterilization necessary?

Generally we do not experience many infections working with axolotls, regardsless of using sterilie equipment or not. We do clean and sterilize equipment at our lab, and we use surgical paper towels. Added to step 1.1.4 “sterile”

4) Provide the exact product names for references. (ligh, electromagnet, etc)

As the editor does recommend against commercial names, we do not act upon this suggestion.

7) no antibiotics?

We do not routinely administer antibiotics in non-implant surgery.

Animal size should be important factor. Provides a recommended animal size.

Adressed above.

Editor's comment:
2.2. and 2.3 are essentially the same. Please check remvoe the redundancy.

Answer:

Step 2.2. changes to include both. Furthermore the 20 minute check has been removed:

"2.2. Check for signs of general anesthesia within 30-45 minutes. These are complete lack of gill movements, righting reflex and no response to either tactile or painful stimuli (gentle pinching of toe web)."

Editor's comment:

Line 223: Please include a citation for this sentence.

Answer:

Citation 10. Diaz Quiroz et. Al added.

Editor's comment:

Results for this part?

Answer:

We are currently in the process of publishing a research paper including the ultrasonic data on spinal cord regeneration and are thus reluctant to include all results on this here. Instead, we have included a figure (Fig. 5) with representative ultrasound results

Added to the results section:

"Ultrasonographic images of the injured spinal cord can be obtained using the above protocol. We found that visualizing the SCI site was possible, due to the obvious lack of bony spinous processes (**Figure 5**). Furhtermore using B-mode we found that we could visualize the dorsal artery of the uninjured spinal cord. This gave us a surrogate marker of integrity. Results are currently being published elsewhere."

Editor's comment:

Details about Figure 5 is missing from the representative result section.

Answer:

see above

Editor's comment:

Missing Figure
Answer:

Figure 6 added to line 352

And legend added:

Figure 6: MRI scans at different time points after injury or sham. CSF surrounding the spinal cord is lacking especially at 3WPI for the SCI animal indicating swelling of the spinal cord. Darkening of the spinal cord indicates edema as well. Notice how these changes disappear as regeneration progresses. Yellow arrow: the area of laminectomy. Figure was originally published by Thygesen et al. in Experimental and Therapeutic Medicine13.

Editor's comment:

How many animals were studied in this case?
Answer:

Sham n = 5, SCI n = 6. In Legend for figure 4.

Editor's comment:

Please include a legend for the video described in the result.
Answer:

"Video showing the neurological function after tactile stimuli and later a nociceptive stimuli. First a healthy control animal, and then an animal suffering from SCI." Added to supplementary video 1 legend.

Editor's comment:

Please include a citation for the same if in quotes. Else, please reword it in your original language.

Answer:

Reference 17 added.

Editor's comment:

Checking that the yellow marks are less than 2.75 pages.

The yellow marked paragraphs corresponds to 2.1 pages

Molecular Medicine Reports
Experimental and Therapeutic Medicine
Oncology Letters
Biomedical Reports
Molecular and Clinical Oncology
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May 21, 2019

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From: Mathias Thygesen [mailto:matthy@clin.au.dk]
Sent: Sunday, May 19, 2019 12:55 PM
To: contact@spandidos-publications.com
Subject: Reprinting figures from article

Dear Spandidos,

I have been invited by the Journal of Visualized Experiments to submit a video manuscript encompassing the surgical method involved in an axolotl model, which we have published work on in Experimental Therapeutic Medicine.
My question is whether I can reuse some of the figures from our article, in that new article.

1. Thygesen MM, Lauridsen H, Pedersen M, Mikkelsen TW, Orlowski D, Rasmussen MM. A clinically relevant blunt spinal cord injury model in the regeneration competent axolotl (*Ambystoma mexicanum*) tail, Experimental and Therapeutic Medicine, https://doi.org/10.3892/etm.2019.7193.

Best regards

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