Controlled semi-automated laser-induced injuries for studying spinal cord regeneration in zebrafish larvae

TITLE:

Controlled Semi-Automated Lased-Induced Injuries for Studying Spinal Cord Regeneration in Zebrafish Larvae

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SUMMARY:

The present protocol describes a method to induce tissue-specific and highly reproducible injuries in zebrafish larvae using a laser lesion system combined with an automated microfluidic platform for larvae handling.

ABSTRACT:

Zebrafish larvae possess a fully functional central nervous system (CNS) with a high regenerative capacity only a few days after fertilization. This makes this animal model very useful for studying spinal cord injury and regeneration. The standard protocol for inducing such lesions is to transect the dorsal part of the trunk manually. However, this technique requires extensive training, and damages additional tissues. A protocol was developed for laser-induced lesions to circumvent these limitations, allowing for high reproducibility and completeness of spinal cord transection over many animals and between different sessions, even for an untrained operator. Furthermore, tissue damage is mainly limited to the spinal cord itself, reducing confounding effects from injuring different tissues, e.g., skin, muscle, and CNS. Moreover, hemi-lesions of the spinal cord are possible. Improved preservation of tissue integrity after laser injury facilitates further dissections needed for additional analyses, such as electrophysiology. Hence, this method offers precise control of the injury extent that is unachievable manually. This allows for new experimental paradigms in this powerful model in the future.

INTRODUCTION:

In contrast to mammals, zebrafish (*Danio rerio*) can repair their central nervous system (CNS) after injury[1]. The use of zebrafish larvae as a model for spinal cord regeneration is relatively recent. It has proven valuable to investigate the cellular and molecular mechanisms underlying repair[2]. This is due to the ease of manipulation, the short experimental cycle (new larvae every week), the tissues' optical transparency, and the larvae's small size, ideally suited for *in vivo* fluorescence microscopy.

In the case of spinal cord regeneration, two additional advantages of using larvae are the speed of recovery, a few days compared to a few weeks for adults, and the ease of inducing injuries using manual techniques. This has been successfully used in many studies[3,4,5], including recent investigations[6,7]. Overall, this leads to increased meaningful data production, high adaptability of experimental protocols, and decreased experimental costs. The use of larvae younger than 5 days post-fertilization also reduces the use of animals following the 3R principles in animal research[8].

After a spinal cord injury in zebrafish larvae, many biological processes occur, including inflammatory response, cell proliferation, neurogenesis, migration of surviving or newly generated cells, reformation of functional axons, and a global remodeling of neural processes circuits and spine tissues[6,7,9,10]. To be successfully orchestrated, these processes involve a finely regulated interaction between a range of cell types, extracellular matrix components, and biochemical signals[11,12]. Unravelling the details of this significant reorganization of a complex tissue such as the spinal cord requires the use and development of precise and controlled experimental approaches.

The primary experimental paradigm used to study spinal cord regeneration in zebrafish is to use surgical means to induce tissue damage by resection, stabbing, or cryoinjury[3,13]. These approaches have the disadvantage of requiring specific training in microsurgery skills which is time-consuming for any new operator and may prevent their use in short-term projects. Furthermore, they usually induce damage to the surrounding tissues, which may influence regeneration.

Another approach is to induce cell damage chemically[14] or by genetic manipulations[15]. The latter allows for highly targeted damage. However, such a technique requires long preparatory work to generate new transgenic fish before doing any experiment, renewed each time a unique cell type is targeted.

There is thus the need for a method allowing targeted but versatile lesions suitable to a variety of studies in regeneration. A solution is to use a laser to induce localized damage in the tissue of interest[16,17,18,19,20]. Indeed, the use of laser-induced tissue damage presents a robust approach for generating spinal cord lesions with many advantages. The microscopes equipped with such laser manipulation modules allow specifying a customized shaped area where cell ablation will occur, with the extra benefit of temporal control. The size and position of the lesion can be thus adapted to address any questions.

The missing feature of most laser lesion systems is the possibility to induce injuries in a highly reproducible way for a series of larvae. Here an original protocol is described using a UV laser to induce semi-automated precise and controlled lesions in zebrafish larvae based on a microfluidic platform designed for automated larvae handling[21]. Moreover, in the system presented here, larvae are inserted in a glass capillary which permits free rotation of the animal around its rostrocaudal axis. The user can choose which side of the larva to present to the laser while allowing fluorescence imaging to precisely target the laser beam and assess the damage after the lesion.

The protocol described here is used with a semi-automated zebrafish larvae imaging system combined with a spinning disk equipped with a UV laser (designated hereafter as the VAST system). However, the main points of the protocol and most of the claims of the technique are valid for any system equipped with a laser capable of cell ablation, including two-photon laser scanning microscopes, spinning-disk microscopes provided with a UV laser (FRAP module), or video-microscopes with a laser module for photo manipulation. One of the main differences between the VAST system and conventional sample handling will be that for the latter, mounting larvae in low-melting-point agarose on glass coverslips/glass-bottom Petri dishes in place of loading them in a 96-well plate will be required.

The benefits offered by this method open opportunities for innovative research on the cellular and molecular mechanisms during the regeneration process. Moreover, the high data quality allows for quantitative investigations in a multidisciplinary context.

PROTOCOL:

All animal studies were carried out with approval from the UK Home Office and according to its regulations, under project license PP8160052. The project was approved by the University of Edinburgh Institutional Animal Care and Use Committee. A schematic of the protocol using the automated zebrafish larvae handling platform is shown in **Figure 1**. All custom software, scripts, and detailed experimental protocols used in this work are available at https://github.com/jasonjearly/micropointpy/.

1. Sample preparation

1.1. At 5 hours post-fertilization, sort the embryos (cf Suppl. Files) for the correct developmental stage[21]. Discard dead eggs, poorly developed, and overdeveloped embryos.

1.2. At 3 days post-fertilization (dpf), anesthetize larvae by adding 2 mL of 0.4% aminobenzoic-acid-ethyl methyl-ester to 50 mL of fish facility water in a 90 mm Petri dish (see **Table of Material**). Use animals raised with phenylthiourea (PTU) to prevent skin pigmentation if it is an issue, which is not the case for spinal cord injuries on 3 dpf larvae described in this protocol.

NOTE: This relatively high anesthetic concentration is used to prevent movements of the larvae following the laser impact.

1.3. Screen the embryos for fluorescent reporter expression (cf Suppl. Files).

NOTE: A fluorescent reporter for the spinal cord (or other structure of interest) is often required to assess the efficiency of the injury. The use of tg(Xla.Tubb:DsRed) helps to identify the spinal cord.

1.4. Transfer the selected larvae into a 96-well plate for use in the VAST system (see **Table of Materials**) with 300 µL of fish facility water per well. Use the medium containing the anesthetic from the 90 mm Petri dish directly. Ensure to have only one larva per well. Prepare one extra empty 96-well plate to collect the lesioned larvae.

NOTE: If using another laser lesion system, mount the larvae in 1% Low-Melting Point (LMP) agarose gel in an appropriate observation chamber.

2. Microscope preparation

2.1. Switch on all the system components (VAST, microscope, laser, PC), including the laser for ablation.

2.2. Once the hardware is fully initialized, launch the microscope software, ImageJ/Fiji, a python integrated development environment (IDE), and the automated zebrafish imaging (VAST system) software if using this platform (see **Table of Materials**).

2.3. Set up the VAST software following the steps below.

2.3.1. When the VAST software launches, choose "Plate" on the first window and click **Done** button (**Figure 2A**). Another small window will pop up asking if the capillary is empty and clean. Verify by looking at the image of the capillary if there are any air bubbles inside. If not, click **Yes**. If there are any bubbles, click **No** and follow step 2.3.2-2.3.3 (**Figure 2B**).

2.3.2. On the Large Particle (LP) Sampler window, click **Prime** to remove air bubbles (**Figure 2C**).

2.3.3. Go to the main software window (with the capillary image) and right-click on the image. Select **Record empty capillary image** on the pop-up menu (**Figure 2B**).

2.3.4. In the LP Sampler window, go to the File menu and select the **Open script** option. Choose a file containing the script corresponding to the experiment to be performed.

2.3.5. In the main VAST software window, go to "File" and choose **Open experiment**. Choose the experiment file corresponding to the planned experiment.

NOTE: Ensure that the boxes "Auto unload" and "Bulk output to waste" are NOT checked.

2.4. Set up the microscope software for imaging.

2.4.1. Launch the microscope imaging software Zen Blue (see **Table of Materials**) to initialize the hardware. This may take a few minutes, depending on the system.

2.4.2. Go to the acquisition settings and set up the microscope for imaging the fluorophore expressed in the larvae. Use a 10x NA 0.5 water-dipping objective to ensure the focal volume is elongated enough along the optical axis to lesion the whole depth of the spinal cord or the targeted tissue.

2.5. Set up ImageJ/Fiji for laser lesions.

2.5.1. Go to the "File" menu, choose **New/Script** to open the script window.

2.5.2. In the "New" window, go to the **File** menu and choose **Open** to load the laser lesion script. ("Manual_MP_Operation.ijm").

2.6. Set up the Python IDE.

2.6.1. Launch the Python IDE.

2.6.2. Go to the "File" menu and choose **Open file** to load the script to manage the laser. ("Watch_for_ROIs_py3.py").

2.6.3. Go to the "Run" menu and choose **Run without Debugging** to run the script. Check that a sequence of messages in the TERMINAL panel appears along with some noise while the laser attenuator initializes (**Figure 2D**).

3. Performing laser lesions on the VAST system

3.1. Center the capillary relative to the microscope objective by moving the stage by clicking on the arrow buttons on the main window of the VAST software (**Figure 2B**).

3.2. Focus on the top of the capillary by looking through the eyepieces and using the transmitted light of the microscope.

CAUTION: The capillary is very fragile and may break if touched by the objective. Move the microscope knob slowly when focusing in and out.

3.3. Place the 96-well plates on the plate holder of the LP Sampler of the VAST system. Place the plate containing larvae on the left holder and the plate for collection on the right. Ensure that the plates are correctly oriented: the A1 well must be in the front left corner of the holder.

3.4. In the VAST software, on the "LP Sampler" window, click on the **Plate template** button and select all the wells containing larvae. Click on the **OK** button to validate and close the window (**Figure 2C**).

3.5. In LP Sampler window, click on the **Run plate** button to start loading a larva.

NOTE: After some time, the larva should be visible in the capillary at position (predefined in the experiment definition file), allowing to injure the spinal cord. The VAST tray light will turn off after a few rotations to set the larva with the lateral side facing the microscope objective.

3.6. Go to the microscope software and click on the **live** button to image the larva.

3.7. Turn the microscope focus knob until the spinal cord central canal is visible.

NOTE: It can be easier to focus using transmitted light first, then refine with fluorescence.

3.8. Take a snapshot in fluorescence and save the image to a dedicated folder.

3.9. Open the image in ImageJ and adjust the contrast if required (using the **Image/Adjust/Brightness/contrast…** menu in ImageJ).

3.10. Click on the **region of interest (ROI)** line tool and draw a short line (20 µm) centered on the spinal cord (**Figure 3A**).

3.11. Switch the microscope to the 100% reflective mirror position.

3.12. Load the ImageJ script and click on the **Run** button. Use the following parameters: Repetition – 2; Sample – 1; Width - 40-micron; Attenuation - 89 (Full laser power) (**Figure 3C**).

3.13. When the laser shot sequence is finished, switch to fluorescence imaging on the imaging software and adjust the focus if required.

NOTE: A shift in focus is often observed due to tail displacement during laser exposure.

3.14. Take a new snapshot and save it.

3.15. Open this new image in ImageJ and draw a new line that should be larger than the spinal cord itself (~80 µm), starting below the ventral side of the spinal cord in the upper part of the notochord and going towards the dorsal side to end in the space between the spinal cord and the skin (**Figure 3B**).

3.16. Switch the microscope to the 100% reflective mirror position.

3.17. Go to the ImageJ script window and click on the **Run** button. Use the following parameters: Repetition – 2; Sample – 1; Width – 40 microns; Attenuation - 89 (Full laser power).

3.18. After the (longer) laser shot sequence is finished, verify the transection quality by imaging fluorescence and moving the focus. Ensure that no cell or axons remain intact in the lesion site, which should appear as a dark or as a faint and homogeneous fluorescent area (**Figure 3D**, bottom panel).

3.19. Collect the lesioned larvae into the empty 96-well plate (with the same well co-ordinates the original well) by going to the main VAST software window and clicking on the **Collect** button.

3.20. Switch back on the VAST system light by clicking the check box **tray light** on the bottom left of the window.

3.21. Repeat step 3.3-3.17 for each new larva to be injured.

4. Post-lesion handling and additional experiments

4.1. Take out larvae from the 96-well plate as soon as possible and transfer them to a clean Petri dish with fresh fish facility water for the larvae to recover post-lesion. Put the Petri dish in an incubator at 28 °C.

NOTE: The damage often continues to propagate in the first hour after the lesion. The actual extent of the lesion should thus be assessed by fluorescence imaging after a delay of approximately one hour.

5. Troubleshooting

5.1. If air bubbles are present in the tubes and capillary of the VAST system, click on the **Prime** button on the LP Sampler window to remove them.

5.2. Consider the unsuccessful lesions (as assessed from the remaining fluorescence in the lesion site, apart from the expected residual and homogenous background), which can be due to several reasons mentioned below.

5.2.1. Low laser power. When this happens, try with a higher value.

NOTE: The VAST system is equipped with a dye laser. This implies that the concentration of the dye solution used for laser light generation can change with time, leading to a decrease in laser power. Replacing with a fresh solution usually solves the problem (see manufacturer's instructions[22]).

5.2.2. Poor calibration. When this happens, verify the calibration and power of the laser system as per step 5.2.2.1-5.2.2.4. If not calibrated correctly, the laser won't be directed to the desired

location, thus leading to unsuccessful lesions or undesired damage in adjacent tissues.

5.2.2.1. Place a mirror slide on top of the capillary chamber. Focus on the coated side (it should face the objective). Use a previous default in the slide to focus more easily.

5.2.2.2. Apply a pattern of laser ablation using a calibration script.

5.2.2.3. Assess the quality of the pattern. The spots or lines should appear sharp and not blurry.

5.2.2.4. Use a ramp with increasing power to evaluate if the laser power has changed compared to the previous sessions.

5.2.3. Larval movement during lesions. Larvae respond differently to anesthesia; thus, the laser lesion may trigger movements of the tail during the process, thus preventing a successful transection. When this happens, take an extra iteration of the laser lesion steps to complete it while still avoiding damage to the surrounding tissues.

5.2.4. Bad focus. When this occurs, focus on the middle of the central canal to get the best results.

5.2.5. ROI drawing, position, and size. The position and size of the ROI are critical for successful transections. The ROI should be larger than the spinal cord and centered on the center of the spinal cord. To solve this, start to draw the ROI from the ventral side of the spinal cord and go up towards the dorsal side to obtain successful transection. This is likely due to tail movements triggered by the sequence of laser shots during the ablation procedure.

REPRESENTATIVE RESULTS:

Validation of spinal cord transection

Structural and functional investigations were performed to assess if the protocol allows a complete spinal cord transection.

First, to verify that the loss in fluorescence at the lesion site was due to neuronal tissue damage and not fluorescence photobleaching from the laser illumination, immunostaining using an antibody against acetylated tubulin (see **Table of Materials)** was performed. A complete disruption of the axons between the caudal and rostral sides of the lesion was observed, confirming the complete transection of the spinal cord (**Figure 4B**). A successful spinal cord transection should not leave any remaining neuronal projection across the lesion site (see **Figure 4C** for an example of an unsuccessful lesion). Using this technique, the success rate of spinal cord laser lesions was estimated to be 75% (4 incomplete transections in 16 animals).

The loss of functionality after laser lesion was investigated using calcium imaging. On intact fish, the spontaneous co-ordinated neuronal network activity generates fluorescence peaks along the

whole spinal cord. A successful transection would interrupt the propagation of this activity between both sides of the lesion. To control the quality of the spinal cord transection, laser lesions were performed on tg(Xla.Tubb:GCaMP6s) larvae at 3 dpf. After collection in a new multi-well plate, larvae were mildly anesthetized. They were mounted on a glass coverslip in low-melting-point agarose to perform fluorescence time-lapse recordings on a confocal microscope from 3 h post-injury. A loss of activity on the caudal side of the lesion site was observed. Indeed, the quantification of fluorescence shows that spikes due to the fish' spontaneous activity were only present on the rostral side after injury but occurred in a co-ordinated manner in the equivalent rostral and caudal positions in intact fish (**Figure 4D,E**). The low residual signal on the caudal side after injury was likely due to the activity of sensory neurons (probably Rohon-Beard sensory neurons on the caudal side of the spinal cord[23]) in reaction to the tail movement induced by muscles contraction on the rostral side.

Regeneration processes induced by laser lesions

After 24 hours post-injury (hpi), the wound started to close, leading to a partial restoration of the initial structure of the spinal cord after 48 h (**Figure 5D**). Using calcium imaging, a partial functional reconnection was confirmed (**Figure 5E,F**) after 48 hpi. The calculation of the ratio (named Connectivity Restoration Index by the authors) between the amplitude of the spikes in the caudal area and the rostral area (**Figure 5G**), showed an increase between 3, 24, and 48 hpi, as expected during spinal cord regeneration.

Laser lesions trigger an immune response

Macrophage (mpeg1:GFP + cells) recruitment was observed after laser lesions using tg(Xla.Tubb:DsRed ;mpeg1:GFP) larvae laser lesions (**Figure 5H,I**). This is consistent with previous studies by the authors using manual lesions demonstrating the essential role of macrophages for successful regeneration of the spinal cord in zebrafish larvae[6,24]. This observation indicates that immune reactions can be studied after laser injury and corroborates that tissue damage occurred.

Laser lesions and manual lesions trigger increased neurogenesis in the spinal cord

Previous studies have used manual lesions to study the neurogenesis that occurs following a spinal cord injury[6,15]. Laser lesions could be a valuable tool to study this phenomenon. A previously published experiment showed increased neurogenesis following a manual spinal cord injury compared to unlesioned controls[15]. Here tg (mnx1:gfp) fish were used as motor neurons are fluorescently labeled. Anti-GFP antibody staining was used to improve the visibility of GFP in the larvae. This was combined with EdU staining[25], which labels newly generated neurons. EdU was added immediately following an injury at 3 dpf, meaning that any cells labeled with EdU were generated post-injury. Therefore, cells that display colocalized staining represent new motor neurons that are born after spinal cord injury. The number of colocalized cells on either side of the injury site, or in an area corresponding to the location and size of the injury site in unlesioned controls (captured in two 50 µm windows) were counted, and the difference in the mean numbers of colocalized cells was analyzed using a one-way ANOVA[26].

This protocol was used on manually, and laser lesioned larvae, to compare the effects of each lesion method on neurogenesis (**Figure 6**). No difference was observed in the number of labeled cells between manual and laser lesions. Unlesioned fish displayed fewer double-labeled cells than lesioned fish in both lesion conditions (**Figure 6D**). This is consistent with previous findings showing increased neurogenesis in manually lesioned fish compared to unlesioned fish[15].

These results support the calcium imaging and acetylated tubulin staining results, as the laser injury elicits a regeneration response comparable to a manual lesion. This indicates that the laser lesion is not simply bleaching the fluorescence in the cells but results in an injury that triggers the same cellular responses that a manual lesion does.

Laser lesions result in less skin and muscle damage than manual lesions

Manual lesions often result in large amounts of muscle and skin damage. In contrast, laser lesions can be targeted more specifically to the spinal cord, reducing the damage to other tissues. To illustrate this, Tg(beta-actin:utrophin-mCh) larvae were used to perform manual and laser lesions. This line fluorescently labels an F-actin-binding protein, allowing the visualization of spinal cord cells and muscle fibers. The larvae were then live mounted and imaged (**Figure 7A,B**). **Figure 7A** shows the damage to the spinal cord. The lack of utrophin in the injury site in both laser and manual lesion conditions suggests that both lesion methods have damaged the cells in the spinal cord. **Figure 7B** shows the muscle damage. There is a clear chevron-like structure to the myotomes in the unlesioned condition, and bundles of actin fibers are visible. There is a visible disruption to the myotome shape in the manual lesion condition, and fewer actin fibers are present. This demonstrates significant muscle damage. However, in the laser lesion condition, the chevron structure of the myotome is maintained. There is some damage to muscle fibers, but this is contained within one or two myotomes compared to within four in the manual lesion condition. In addition, there is minor skin damage in the laser lesion condition compared to the manual lesion condition, as shown in images taken on a stereo microscope in **Figure 7C**.

Altogether, these results demonstrate that reproducible, semi-automated laser lesions have the potential to be a powerful tool to study neural regeneration in zebrafish.

DISCUSSION:

There is an urgent need for a deeper understanding of the processes at play during regeneration in zebrafish. This animal model offers many benefits for biomedical research, in particular for spinal cord injuries[1]. Most of the studies involve manual lesions that require a well-trained operator and induce multi-tissue damage. A laser lesion protocol is presented here, allowing control over the lesion characteristics and reduced damage to the surrounding tissues. Furthermore, this technique is easy enough to be successfully used by relatively untrained experimenters.

Critical steps in the protocol are the calibration of the laser and the definition of ROIs. In practice, the calibration is very stable (even for months), and once the right size and position of the ROI have been determined, the use of this technique is straightforward. Although the protocol

described how to perform the lesions on specific equipment, most of the benefits of laser lesions are available for different systems, such as a spinning-disk microscope.

The main limitations of this protocol are the need to use a fluorescence reporter of the spinal cord and the time required to perform the lesions (~5 min/fish). The latter is compensated for by high reproducibility requiring fewer animals. However, manual lesions are still viable for applications such as drug testing where many lesioned animals are needed. As shown here, the extent of lesion-induced neurogenesis is comparable between laser and manual lesions.

However, laser injury has enormous potential applications, some of them related to the unique benefits offered. For example, a rotating capillary allows performing lesions in a large variety of positions in a controlled way. For example, it could be used to induce single neuron axotomy in Mauthner cells (data not shown), as has also been demonstrated in the work of Bhatt et al.[15]. This would not be possible using manual lesions.

The results also demonstrate that the damage is mainly contained to the spinal cord, with minimal damage to surrounding tissues. This could mean that cellular responses seen following a laser lesion are more likely to be attributed to the spinal cord specifically rather than signaling from other damaged tissues. It also could mean that laser lesioned larvae are more able to withstand further preparations for experiments. For example, dissection for electrophysiology involves removing the trunk skin using forceps[27,28,29], which would result in high mechanical pressure placed on the already delicate injury site and risk any axonal connections to be broken again. The integrity of skin and muscle tissue seen in laser-lesioned larvae could protect the lesion site from further damage and result in a more accurate representation of the level of regeneration achieved.

Moreover, the improved localization of damage after laser injury limits the extension of coupling between different regeneration processes, which may mask more subtle processes when using manual lesions. The approach to experimental injury in the larval zebrafish described here may open a range of new investigations in the context of quantitative biology, biological physics, and computational biology.

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DISCLOSURES:
The authors have nothing to disclose.

FIGURE LEGENDS:

Figure 1: Schematic of the semi-automatic laser-injury workflow. 3 days post-fertilization (dpf), larvae are loaded into a 96-well plate and placed on the automated larvae handling platform. Then each larva is loaded into a capillary placed under a 10x NA 0.5 lens on an upright microscope for imaging and laser lesion. After lesions, larvae are unloaded to a new 96-well plate for collection and further experiments. On the top, transmitted and fluorescence images of tg (Xla.Tubb:DsRed) 3 dpf larvae before and after laser lesion (scale bar = 50 µm). Larvae are oriented rostral left and dorsal up (for all figures).

Figure 2: Software start-up for the semi-automated zebrafish larvae imaging system and laser control system. (**A**) VAST software at start-up. (**B**) The main window of the VAST software shows the empty capillary. (**C**) LP Sampler window with a blank plate template. (**D**) The view of python IDE with the Watch_for_ROIs_py3.py script running. The orange rectangle points out the terminal tab with messages displayed during the initialization of the laser attenuator.

Figure 3: Example of laser lesion sequence on tg(Xla.Tubb:DsRed) 3 dpf larvae. (**A**) The first step of laser lesion using a 20 µm line after selecting the line ROI tool from the ImageJ toolbar. (**B**) Second step with an 80 µm line for complete transection of the spinal cord. (**C**) View of the script used for controlling the laser from ImageJ. (**D**) The sequence of images during laser lesions. Top: Before lesion; Middle: immediately after the first step; Bottom: immediately after the second step (scale bar = 50 µm).

Figure 4: Acetylated tubulin immunostaining (A-C) and calcium imaging (D,E) indicate that laser lesion entirely disrupts the continuity of spinal tissue. (**A**) Intact spinal cord. (**B**) Complete transection of the spinal showing a complete disruption of the spinal cord tissue along both the dorsal-ventral and medial-lateral axes. (**C**) Incomplete transection. (scale bar = 50 µm). (**D**) Transected spinal cord on a tg(Xla.Tubb:GCaMP6s) 3 dpf larva. The rectangles show the ROIs used to quantify the fluorescence intensity in the lesion's rostral (blue) and caudal (orange) sides. (**E**) Graph of the fluorescence intensity changes over time in the rostral and caudal analysis ROIs.

Figure 5: Laser injury elicits an immune response and leads to successful anatomical and functional recovery. (**A-D**) The maximum intensity projection fluorescence images of a tg(Xla.Tubb:DsRed) 3dpf larva before (**A**) and at different times after the laser lesion: after 3 h (**B**), after 24 h (**C**), and after 48 h (**D**). (**E-G**) The use of calcium imaging to assess the function restoration. (**E**) Lesioned tg(Xla.Tubb:GCaMP6s) larva with analysis ROIs. (**F**) Graph of the fluorescence intensity changes over time in the rostral and caudal analysis ROIs. (**G**) Quantification of the ratio between caudal and rostral spike amplitudes (Connectivity Restoration Index) at 3, 24, 48 h post-lesion (N = 3). (**H-I**) Characterization of the immune response after lesion. (**H**) Fluorescence images of unlesioned (left) and lesioned (right) tg(Xla.Tubb:DsRed;mpeg1:GFP) 3 dfp larva showing the accumulation of macrophages (mpeg1+ cell, green) at 6 hpi. (**I**) Quantification of the number of macrophages at 6 h post-lesion in injured and intact larvae (N = 3) (scale bars = 50 µm).

Figure 6: Lesion-induced generation of motor neurons is comparable between laser and manual lesion. (**A-C**) Images from the ApoTome microscope of tg(mnx1:gfp) 5 dpf larvae with EdU staining, in laser lesion (**A**), manual lesion (**B**), and unlesioned (**C**) conditions. Arrowheads denote cells double-labeled for both markers. Scale bar = 100 µm. (**A'- C'**) Higher magnification of double-labeled cells denoted by white boxes. (**D**) Quantification of cell counts for the number of colocalized cells in each larva. 50 µm windows were placed on either side of the injury site, and colocalized cells were counted in all Z-stack images. One-way ANOVA was performed with Tukey's posthoc test[30]. No significant difference between laser and manual lesions ($p = 0.909$). Significantly fewer mnx1:gfp+/EdU+ cells in unlesioned controls compared to laser lesion (2.4 fold change, $p = 0.011$) and manual lesion (2.3 fold change, $p = 0.018$).

Figure 7: Laser lesion induces less muscle and skin damage than the manual lesion. (**A-B**) Single Z-stack images of tg(beta-actin:utrophin-mCherry) 3 dpf larvae in the unlesioned, manual lesion and laser lesion conditions, taken on the confocal microscope at 20x magnification. White arrows denote the injury site. Scale bar = 50 µm. (**A**) denotes Z-stacks where the spinal cord and notochord are visible. SC labels the spinal cord, and NC labels the notochord. (**B**) denotes Z-stacks where the muscle fibers are visible. (**C**) Images were taken on the stereo microscope of 3 dpf larvae in the unlesioned, manual lesion, and laser lesion conditions. Larvae were pinned to a platform using tungsten wire pins (visible in laser lesion image). The black box denotes the lesion site. Scale bar = 50 µm.

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