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Stab Wound Injury Model of the Adult Optic Tectum using Zebrafish and Medaka for the Comparative Analysis of Regenerative Capacity

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TITLE:

Stab Wound Injury Model of the Adult Optic Tectum using Zebrafish and Medaka for the Comparative Analysis of Regenerative Capacity

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SUMMARY:

A mechanical brain injury model in the adult zebrafish is described to investigate the molecular mechanisms regulating their high regenerative capacity. The method explains to create a stab wound injury in the optic tectum of multiple species of small fish to evaluate the regenerative responses using fluorescent immunostaining.

ABSTRACT:

While zebrafish have a superior capacity to regenerate their central nervous system (CNS), medaka has a lower CNS regenerative capacity. A brain injury model was developed in the adult optic tectum of zebrafish and medaka and performed comparative histological and molecular analyses to elucidate the molecular mechanisms regulating the high regenerative capacity of this tissue across these fish species. Here a stab wound injury model is presented for the adult optic tectum using a needle and histological analyses for proliferation and differentiation of the neural stem cells (NSCs). A needle was manually inserted into the central region of the optic tectum, and then the fish were intracardially perfused, and their brains were dissected. These tissues were then cryosectioned and evaluated using immunostaining against the appropriate NSC proliferation and differentiation markers. This tectum injury model provides robust and reproducible results in both zebrafish and medaka, allowing for comparing NSC responses after injury. This method is available for small teleosts, including zebrafish, medaka, and African killifish, and enables us to compare their regenerative capacity and investigate unique molecular mechanisms.

INTRODUCTION:

Zebrafish (*Danio rerio*) have an increased ability to regenerate their central nervous system (CNS) compared to other mammals[1-3]. Recently, to better understand the molecular mechanisms

underlying this increased regenerative capacity, comparative analyses of tissue regeneration using next-generation sequencing technology have been performed[4-6]. The brain structures in zebrafish and tetrapods are quite different[7-9]. This means that several brain injury models using small fish with similar brain structures and biological features have been developed to facilitate the investigation of the underlying molecular mechanisms contributing to this increased regenerative capacity.

In addition, medaka (*Oryzias latipes*) is a popular laboratory animal with a low capacity for heart and neuronal regeneration[10-13] compared with zebrafish. Zebrafish and medaka have similar brain structures and niches for adult neural stem cells (NSCs)[14-17]. In zebrafish and medaka, the optic tectum includes two types of NSCs, neuroepithelial-like stem cells and radial glial cells (RGCs)[15,18]. A stab wound injury for the optic tectum of adult zebrafish was previously developed, and this model was used to investigate the molecular mechanisms regulating brain regeneration in these animals[19-23]. This young adult zebrafish stab wound injury model induced regenerative neurogenesis from RGCs[19,24,25]. This stab wound injury in the optic tectum is a robust and reproducible method[13,19-25.] When the same injury model was applied to adult medaka, the low neurogenic capacity of RGCs in medaka optic tectum was revealed *via* the comparative analysis of RGC proliferation and differentiation following injury[13].

Stab wound injury models in the optic tectum have also been developed in mummichog models[26], but details of the tectum injury have been not well documented when compared with telencephalic injury[27]. The stab wound injury in the optic tectum using zebrafish and medaka allows the investigation of the differential cellular responses and gene expression between species with differential regenerative capacity. This protocol describes how to perform a stab wound injury in the optic tectum using an injection needle. This method can be applied to small fish like zebrafish and medaka. The processes for sample preparation for histological analysis and cellular proliferation and differentiation analysis using fluorescent immunohistochemistry and cryosections are explained here.

PROTOCOL:

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the National Institute of Advanced Industrial Science and Technology. Zebrafish and medaka were maintained according to standard procedures[28].

1. Stab wound injury in the adult optic tectum

1.1. Prepare a 0.4% (w/v) tricaine stock solution for anesthesia. For a 100 mL stock solution, dissolve 400 mg tricaine methanesulfonate (see **Table of Materials**) in 90 mL of distilled water and adjust the pH to 7.0 using 1M Tris HCl buffer (pH 9.0). Once the pH is adjusted, add water up to 100 mL, then make appropriate aliquots and store them at -20 °C.

1.2. To anesthetize the adult zebrafish or medaka, prepare a 0.02 % (w/v) tricaine solution by diluting the 0.4 % tricaine stock solution with fish facility water.

1.3. Anesthetize the fish with 0.02% tricaine solution; they become anesthetized when they do not move or respond to touch (1-2 min).

1.4. Place the anesthetized fish on a Styrofoam tray (see **Table of Materials**) and fix the fish upright between two 30 G needles inserted vertically into the Styrofoam.

1.5. Hold the fish body to prevent the fish head from moving and vertically insert a 30 G needle through the skull into the medial region of one of the optic tectum hemispheres (**Figure 1A,B**). The insertion depth is ~0.75 mm, almost equal to half the length of the 30 G bevel. This insertion depth induces brain injury on zebrafish and medaka because of the similar body size of these fish.

NOTE: Medaka has scales on their skull. Scratch and remove the scales using the 30 G needle to efficiently and accurately induce the stab wound injury (**Figure 1C**).

1.6. Transfer the injured fish into a fish tank with fresh fish facility water and move the tank to the fish breeding system once they have fully recovered from the anesthesia.

NOTE: Fish will recover and start freely moving within a few minutes.

1.7. For lineage analysis after the injury, prepare fresh fish facility water containing 5 mM bromodeoxyuridine (BrdU) (see **Table of Materials**) and then incubate the fish with this 5 mM BrdU solution for a predetermined amount of time[13,19] (as determined by an experimental design). Then dissect these fish as per step 2 and evaluate BrdU incorporation and cell lineage identity as appropriate.

NOTE: This step is an optional step for cell lineage analysis. To detect BrdU signals, perform antigen retrieval as shown in step 4.3.

2. Brain dissection

2.1. Prepare 0.02% tricaine solution, a Styrofoam tray for dissection, and a 10 mL syringe containing phosphate-buffered saline (1x PBS) with an extension tube and 30 G needles (see **Table of Materials**) for intracardial perfusion.

2.2. Anesthetize the injured fish at selected time points.

2.3. Place paper towels on the Styrofoam tray and place the anesthetized fish on the paper towels.

2.4. Hold the fish body in place by vertically inserting two 30 G needles on either side of the anal fin (**Figure 2A**).

2.5. Make a ventral incision from the anus to the heart (**Figure 2B**) and keep the heart visible by inserting another two 30 G needles on each side of this organ (**Figure 2C**).

NOTE: The heart is covered in the silvery epithelial layer of the hypodermis in both zebrafish and medaka (**Figure 2C**).

2.6. Gently remove the silver epithelial layer with the tip of the forceps (**Figure 2D**).

2.7. Insert the 30 G needle into the ventricle, keeping the bevel up, and make an incision into the atrium using the forceps (**Figure 2E**). Carefully press down on the syringe to push the 1x PBS and confirm that the atrium flushed the blood.

NOTE: To prevent the needle from penetrating the ventricle, carefully insert the needle to the depth of the half bevel and then adjust the insertion depth. If the perfusion is well done, gills turn white (**Figure 2F,G**).

2.8. Stop the perfusion after the blood is drained and the gills turn white.

2.9. Remove the needles fixing the fish body in place and fix the fish ventral side down (**Figure 2H**). Cut the spinal cord and remove the skull from the optic tectum and telencephalon (**Figure 2I**). Then, cut the optic nerves and dissect the brain carefully.

NOTE: Watch out for the optic nerves during the dissection because the nerves are connected to the tectum. When the optic nerve is pulled, the optic tectum can be detached from the brain. If the blood removal is not complete, the brain looks light pink (the right brain in **Figure 2J**).

2.10. Transfer the brains into 1.5 mL tubes with 1 mL of 4 % paraformaldehyde in 1x PBS and fix them overnight at 4 °C.

3. Preparation of frozen sections

3.1. Wash the fixed brains three times in 1x PBS for 5 min each.

3.2. To cryoprotect the brains, transfer them into 1.5 mL tubes with 1 mL of 30 % (w/v) sucrose in 1x PBS and incubate them overnight at 4 °C. Prepare the embedding compound (2:1 mixture of OCT compound and 30% sucrose, see **Table of Materials**) by combining 30 mL of OCT and 15 mL of 30 % sucrose. Store this at 4 °C.

NOTE: To remove the bubbles from the mixed compound, centrifuge the 50 mL tubes at 10,000 x *g* for 2 min at room temperature or place the tubes upright overnight.

3.3. To cool the cryomold (see **Table of Materials**) once, embed the dissected brain in the cryomold with the embedding compound and incubate an aluminum block overnight at -80 °C.

NOTE: Liquid nitrogen can also be used to cool the aluminum block. In that case, the aluminum block should be placed in a Styrofoam box filled with enough liquid nitrogen to cover the

aluminum block just before embedding. After confirming the liquid nitrogen sublimation, place the cryomold on the precooled aluminum block. It is better to confirm how many cryomolds can be placed on the aluminum block at once. It is advisable to use liquid nitrogen or an additional precooled block to ensure enough space to cool all samples quickly.

3.4. Put the precooled aluminum block in a Styrofoam box. Use a Styrofoam box with a lid to prevent the aluminum block from warming.

3.5. For coronal sections, embed the brain in a cryomold and adjust the orientation using forceps under the microscope (**Figure 3A**).

3.6. Place the cryomold on the precooled aluminum block in the Styrofoam box and allow the OCT compound to freeze (**Figure 3B**). When the OCT compound starts to freeze, it becomes white.

3.7. Apply a small circle of OCT compound on a specimen disc, and mount the cryoblock to attach the cryoblock to the specimen disc.

3.8. Place the specimen disc in the cryostat and freeze the OCT compound completely.

3.9. Place the specimen disc on the specimen head in the cryostat.

3.10. Set the orientation of the cryoblock and trim the OCT to remove any extra regions.

NOTE: Adjust the orientation of the cutting plane while cutting the telencephalon. The blade is equipped with a cryostat. It is possible to adjust the orientation of the cutting plane by changing the angle of the specimen head.

3.11. Cut 14 µm thick serial sections through the whole optic tectum using a cryostat. Store the slides at -25 °C.

NOTE: Long-term storage should be at -80 °C.

4. Fluorescent immunostaining

4.1. Dry glass slides in a slide rack for 30 min at room temperature (RT). Wash slides in 1x PBS for 30 min at RT. If immunostaining with anti-PCNA or BrdU antibodies is to be performed, proceed to step 4.2 or step 4.3.

4.2. Prepare 500 mL of 10 mM sodium citrate in a 500 mL beaker and warm the citrate buffer to 85 °C on a hot plate magnetic stirrer, then place the slides in a slide staining rack and incubate the rack in the citrate buffer at 85 °C for 30 min. To avoid boiling, keep the temperature around 85 °C. After antigen retrieval, wash the slides three times in 0.1 % Triton in 1x PBS (PBSTr), with each wash taking 5 min at RT.

NOTE: This step is an optional step for proliferating cell nuclear antigen (PCNA) retrieval.

4.3. Prepare a 2 N HCl solution by diluting 12 N HCl in distilled water. Using a liquid blocker (see **Table of Materials**), draw a line as a hydrophobic barrier to keep the 2 N HCl solution around the sections.

4.3.1. Place the slides on immunostaining trays and apply 200-300 µL of the 2N HCl solution. Incubate the trays at 37 °C for 30 min. After antigen retrieval, wash three times in 0.1 % PBSTr with each wash taking 5 min at RT.

NOTE: This step is an optional step for BrdU antigen retrieval.

4.4. Absorb and remove the remaining solution using paper towels. Then, use a liquid blocker to draw a hydrophobic barrier to keep the antibody solution around the sections as necessary.

4.5. Place the slides on trays and apply 200-300 µL of blocking solution, 3% (v/v) horse serum in PBSTr) to every slide, and incubate the slides for 1 h at RT.

4.6. Wash the sections with PBSTr for 5 min at RT and repeat three times.

4.7. Prepare the primary antibodies (see **Table of Materials**) using the blocking solution (200-300 µL of antibody solution for each slide). Remove the remaining PBSTr with paper towels and place the slides on the immunostaining trays. Apply the primary antibody solution to every slide and incubate trays overnight at 4 °C.

4.8. Wash the sections with PBSTr for 5 min at RT and repeat three times.

4.9. Prepare fluorescent secondary antibody solution (see **Table of Materials**) using the blocking buffer (1:500). Remove the remaining PBSTr with paper towels and place the slides on the immunostaining trays. Apply the secondary antibody solution to every slide and shade trays with aluminum foil. Incubate for 1-2 h at RT.

4.10. Wash the sections with PBSTr for 5 min at RT three times without exposing them to light.

4.11. Prepare Hoechst solution (1:500 dilution in 1x PBS, see **Table of Materials**) for nuclear staining. Remove the remaining PBSTr with paper towels and place the slides on the immunostaining trays. Apply the Hoechst solution to every slide and cover the trays with aluminum foil. Incubate for 30 min at RT.

4.12. Wash the sections with 1x PBS for 10 min at RT without exposing them to light.

4.13. Absorb and remove the remaining 1x PBS using paper towels and mount the sections on slides using a water-soluble mounting medium (see **Table of Materials**) for fluorescent

immunostaining. Slowly set a 24 x 45 mm coverslip onto the sections. Store at 4 °C, without exposing the slides to light.

4.14. Observe and image the sections under fluorescent microscopy or confocal microscopy.

REPRESENTATIVE RESULTS:

Stab wound injury in the optic tectum using needle insertion into the right hemisphere (**Figure 1**, **Figure 4A,** and **Figure 5A**) induces various cellular responses, including radial glial cell (RGC) proliferation and the generation of newborn neurons. Similarly, aged populations of zebrafish and medaka were used to counteract any aging effects in the regenerative response. Then fluorescent immunostaining was performed on the frozen sections, and the RGC proliferation and differentiation were analyzed after the tectum injury in the zebrafish and medaka (**Figure 4-5**)[13].

Antibodies against a proliferating cell marker were used, proliferating cell antigen (PCNA), and an RGC marker, brain lipid-binding protein (BLBP), available in zebrafish and medaka to evaluate the RGC proliferation in these tissues[13,19]. As previously described, most of the RGCs were quiescent (PCNA negative) in the contralateral uninjured hemisphere (**Figure 4B**)[13.] Still, RGC proliferation was induced at 2 days post-injury (dpi) in the medaka tectum (**Figure 4C,D**)[13]. Induction of RGC proliferation after the injury is a common feature of both the zebrafish and medaka regenerative responses[13,19].

BrdU labeling is a simple method used to evaluate cell lineage and analyze RGC differentiation after brain injury (**Figure 5A**)[13]. Immunostaining with antibodies for a pan-neuronal marker, HuC, and BrdU was previously used to compare RGC differentiation in the injured tectum of zebrafish and medaka (**Figure 5B,C**)[13]. If these antibodies are available in the target species, comparative analyses can be performed.

If the injury is appropriately induced, the injury site is located in the central-dorsal region in the optic tectum (**Figure 4C**). Nuclear staining and hematoxylin and eosin staining can then be used to confirm the injury site[13,19-25]. After the injury, a disturbed periventricular gray zone with nuclear staining can be observed (**Figure 4C**). If the injury is located in the medial dorsal region, RGC proliferation is not significantly increased[25].

FIGURE LEGENDS:

Figure 1: Stab wound injury in adult optic tectum using a needle. (**A**) Dorsal view of an adult zebrafish. Zebrafish is kept upright between two bent needles inserted vertically into a Styrofoam. (**A'**) Magnified image of the boxed area in (**A**). 30 G needle is inserted into the medial region of the border between two skulls called *os frontale* and *os parietale* on the optic tectum. The yellow circle indicates the injury site, and white dashed lines indicate the two skulls on the optic tectum. (**B**) Dorsal view of adult medaka. (**B'**) A magnified image of the boxed area in (**B**). 30 G needle is inserted into the border on the optic tectum. The yellow circle indicates the injury site, and white dashed lines indicate two skulls on the optic tectum. (**C**) Medaka has scales on the

skull. Skull scales are to be removed before the stab wound injury. The dashed line indicates the scale on the optic tectum. Scale bar: 2 mm in **A-B**, 1 mm in **A'**, **B'** and **C**. Telencephalon (Tel), optic tectum (OT), *os frontale* (F), and *os parietale* (P).

Figure 2: Intracardiac perfusion in small adult fish. (**A**) Ventral view of a zebrafish fixed on Styrofoam using bent needles ready for intracardiac perfusion and brain dissection. (**B**) A ventral incision is made from the origin of the anal fins to the chest. (**C**) The heart is behind a silvery epithelial layer called the hypodermis in both zebrafish and medaka. Another fixation using a bent needle beside the silver epithelial layer allows for easier access. The solid white line indicates the ventricle (V), and the dotted line indicates the hypodermis. (**D**) The silver epithelial layer is removed before the intracardial perfusion of 1x PBS. (**E-G**) Canula is inserted into the ventricle for intracardiac perfusion. Gills before (**F**) and after (**G**) the intracardiac perfusion. If the blood removal is not complete, the gills remain red. (**H-J**) Brain dissection after PBS perfusion to remove the blood from the tissues. Remove skulls on the optic tectum and telencephalon as shown in (**I**). If the blood removal is not complete, the brain looks light pink (the right brain in (**J**)). Scale bar: 2 mm in **A-C** and **H**, 1 mm in **D-G** and **I-J.** The olfactory bulb (OB), telencephalon (Tel), optic tectum (OT), bulbus arteriosus (Ba), ventricle (V), and atrium (At).

Figure 3: Brain embedding for frozen sections. (**A**) The brain is embedded in a cryomold with an embedding compound. Anterior is down. (**B**) Cryomolds are cooled on a precooled aluminum box. Telencephalon (Tel), optic tectum (OT).

Figure 4: Representative results of fluorescent immunostaining against RGC proliferation after tectum injury in adult medaka. (**A**) Schematic view of the stab wound injury to the right hemisphere of the optic tectum and coronal section. (**B-C**) Representative results of proliferative RGCs (PCNA + BLBP + cells) in the contralateral uninjured (**B**) and injured (**C**) side at 2 days post-injury. White arrowhead in **C'** indicates a disturbed periventricular gray zone by the stab wound injury. (**D**) Magnified images of the boxed area in (**C**). White arrowheads in (**D**) indicate PCNA + BLBP + cells. Scale bar: 50 µm in **B-D**. Adapted with permission from Reference[13].

Figure 5: Representative results of the fluorescent immunostaining for the generation of newborn neurons after tectum injury. (**A**) Schematic view of bromodeoxyuridine (BrdU) treatment and the stab wound injury in the optic tectum and coronal section. (**B-C**) Representative results of newborn neurons (BrdU + HuC + cells) at 7 days post-injury in the injured zebrafish (**B**) and medaka (**C**). Scale bar: 50 µm in **B-C**. Adapted with permission from Reference[13].

DISCUSSION:

Here a set of methods is described which can be used to induce stab wound injuries in the optic tectum utilizing a needle to facilitate the evaluation of RGC proliferation and differentiation after brain injury. Needle-mediated stab wounds are a simple, efficiently implemented method that can be applied to many experimental samples using a standard set of tools. Stab wound injury models for several regions of the zebrafish brain have been developed[3,19,29]. The optic tectum is one of the most significant parts of the brain and is easy to manipulate. Moreover, most RGCs in

the optic tectum are quiescent under physiological conditions when compared to the telencephalon, making it easier to observe RGC proliferation and differentiation depending on the injury[3,19].

One of the critical steps and limitations in stab wound injuries is manual needle insertion; a consistent injury is necessary for creating reproducible results and facilitating comparative analysis. The precise location and depth of insertion are crucial and help create reproducible injuries in experiments. This paper provides clear guidelines for making similar injuries each time. Moreover, the proliferation of RGC after the injury is essential for the neurogenesis of the injured tectum. In injured zebrafish and medaka, RGC proliferation increases at 1 dpi and returns to basal levels, the same as in the contralateral uninjured hemisphere, at 7 dpi[13,19].

Stab wound injury is one of the mechanical injury methods that induce non-specific cell ablation. In contrast, transgenic approaches to cell-specific ablation such as nitroreductase/metronidazole system have also been developed[30-32]. These ablation models should be selected based on the experimental purpose. Non-specific ablation is suitable for brain injuries such as stab wounding and ischemic stroke. In contrast, cell-specific ablation might be more appropriate for evaluating the degeneration of specific cells associated with neurodegenerative diseases such as Parkinson's disease.

Recently, ischemic injury models using zebrafish have been developed[33], but these models need transgenic lines and fluorescent microscopy to monitor blood flow. Therefore, these models are challenging to apply to species with poor genetic approaches, and their throughput is lower than the stab wound injury model.

As mentioned above, stab wound injury in the optic tectum is simple and easily applied in other small fish models such as African killifish and mummichog with common tools[26]. Furthermore, comparative analysis between species has been well investigated using sequencing technology. Therefore, this simple method remains essential when studying the regenerative capacity of NSCs in zebrafish using comparative analysis of cellular responses and gene expression.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., Schachner, M. Axonal regrowth after spinal cord transection in adult zebrafish. *Journal of Comparative Neurology*. **377** (4), 577-595 (1997).
2. Raymond, P. A., Barthel, L. K., Bernardos, R. L., Perkowski, J. J. Molecular characterization of retinal stem cells and their niches in adult zebrafish. *BMC Developmental Biology.* **6**, 36 (2006).

3. März, M., Schmidt, R., Rastegar, S., Strähle, U. Regenerative response following stab injury in the adult zebrafish telencephalon. *Developmental Dynamics.* **240** (9), 2221-2231 (2011).
4. Kang, J., et al. Modulation of tissue repair by regeneration enhancer elements. *Nature.* **532** (7598), 201-206 (2016).
5. Simões, F. C., et al. Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. *Nature Communications.* **11** (1), 600 (2020).
6. Hoang, T., et al. Gene regulatory networks controlling vertebrate retinal regeneration. *Science.* **370** (6519), eabb8598 (2020).
7. Alunni, A., Bally-Cuif, L. A comparative view of regenerative neurogenesis in vertebrates. *Development*. **143** (5), 741–753 (2016).
8. Diotel, N., Lübke, L., Strähle, U., Rastegar, S. Common and distinct features of adult neurogenesis and regeneration in the telencephalon of zebrafish and mammals. *Frontiers in Neuroscience*. **14**, 568930 (2020).
9. Labusch, M., Mancini, L., Morizet, D., Bally-Cuif, L. Conserved and divergent features of adult neurogenesis in zebrafish. *Frontiers in Cell and Developmental Biology*. **8**, 525 (2020).
10. Ito, K., et al. Differential reparative phenotypes between zebrafish and medaka after cardiac injury. *Developmental Dynamics.* **243** (9), 1106-1115 (2014).
11. Lai, S. L., et al. Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. *eLife*. **6**, e25605 (2017).
12. Lust, K., Wittbrodt, J. Activating the regenerative potential of Müller glia cells in a regeneration-deficient retina. *eLife*. **7**, e32319 (2018).
13. Shimizu, Y., Kawasaki, T. Differential regenerative capacity of the optic tectum of adult medaka and zebrafish. *Frontiers in Cell and Developmental Biology*. **9**, 686755 (2021).
14. Adolf, B., et al. Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Developmental Biology*. **295** (1), 278–293 (2006).
15. Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., Brand, M. Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Developmental Biology*. **295** (1), 263–277 (2006).
16. Alunni, A., et al. Evidence for neural stem cells in the medaka optic tectum proliferation zones. *Developmental Neurobiology*. **70** (10), 693-713 (2010).
17. Kuroyanagi, Y., et al. Proliferation zones in adult medaka (Oryzias latipes) brain. *Brain Research.* **1323**, 33–40 (2010).
18. Ito, Y., Tanaka, H., Okamoto, H., Ohshima, T. Characterization of neural stem cells and their progeny in the adult zebrafish optic tectum. *Developmental Biology*. **342** (1), 26-38 (2010).
19. Shimizu, Y., Ueda, Y., Ohshima, T. Wnt signaling regulates proliferation and differentiation of radial glia in regenerative processes after stab injury in the optic tectum of adult zebrafish. *Glia*. **66** (7), 1382–1394 (2018).
20. Ueda, Y., Shimizu, Y., Shimizu, N., Ishitani, T., Ohshima, T. Involvement of sonic hedgehog and notch signaling in regenerative neurogenesis in adult zebrafish optic tectum after stab injury. *Journal of Comparative Neurology*. **526** (15), 2360-2372 (2018).
21. Kiyooka, M., Shimizu, Y., Ohshima, T. Histone deacetylase inhibition promotes regenerative neurogenesis after stab wound injury in the adult zebrafish optic tectum. *Biochemical and Biophysical Research Communications*. **529** (2), 366-371 (2020).
22. Shimizu, Y., Kawasaki, T. Histone acetyltransferase EP300 regulates the proliferation and

differentiation of neural stem cells during adult neurogenesis and regenerative neurogenesis in the zebrafish optic tectum. *Neuroscience Letters*. **756**, 135978 (2021).
23. Shimizu, Y., Kiyooka, M. Ohshima, T. Transcriptome analyses reveal IL6/Stat3 signaling involvement in radial glia proliferation after stab wound injury in the adult zebrafish optic tectum. *Frontiers in Cell and Developmental Biology*. **9**, 668408 (2021).
24. Lindsey, B. W., et al. Midbrain tectal stem cells display diverse regenerative capacities in zebrafish. *Scientific Reports*. **9** (1), 4420 (2019).
25. Yu, S., He, J. Stochastic cell-cycle entry and cell-state-dependent fate outputs of injury-reactivated tectal radial glia in zebrafish. *eLife*. **8**, e48660 (2019).
26. Bisese, E. C., et al. The acute transcriptome response of the midbrain/diencephalon to injury in the adult mummichog (Fundulus heteroclitus). *Molecular Brain*. **12** (1), 119 (2019).
27. Schmidt, R., Beil, T., Strähle, U., Rastegar, S. Stab wound injury of the zebrafish adult telencephalon: a method to investigate vertebrate brain neurogenesis and regeneration. *Journal of Visualized Experiments*. **4**, e51753 (2014).
28. Westerfield, M. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio) (5th ed.). Esugene: University of Oregon Press (2007).
29. Kaslin, J., Kroehne, V., Ganz, J., Hans, S., Brand, M. Distinct roles of neuroepithelial-like and radial glia-like progenitor cells in cerebellar regeneration. *Development*. **144** (8), 1462-1471 (2017).
30. Curado, S., et al. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Developmental Dynamics*. **236** (4), 1025-1035 (2007).
31. Shimizu, Y., Ito, Y., Tanaka, H., Ohshima, T. Radial glial cell-specific ablation in the adult zebrafish brain. *Genesis*. **53** (7), 431-439 (2015).
32. Godoy, R., et al. Dopaminergic neurons regenerate following chemogenetic ablation in the olfactory bulb of adult zebrafish (Danio rerio). *Scientific Reports*. **10** (1), 12825 (2020).
33. Sawahata, M., Izumi, Y., Akaike, A., Kume, T. In vivo brain ischemia-reperfusion model induced by hypoxia-reoxygenation using zebrafish larvae. *Brain Research Bulletin*. **173**, 45-52 (2021).

Figure 1

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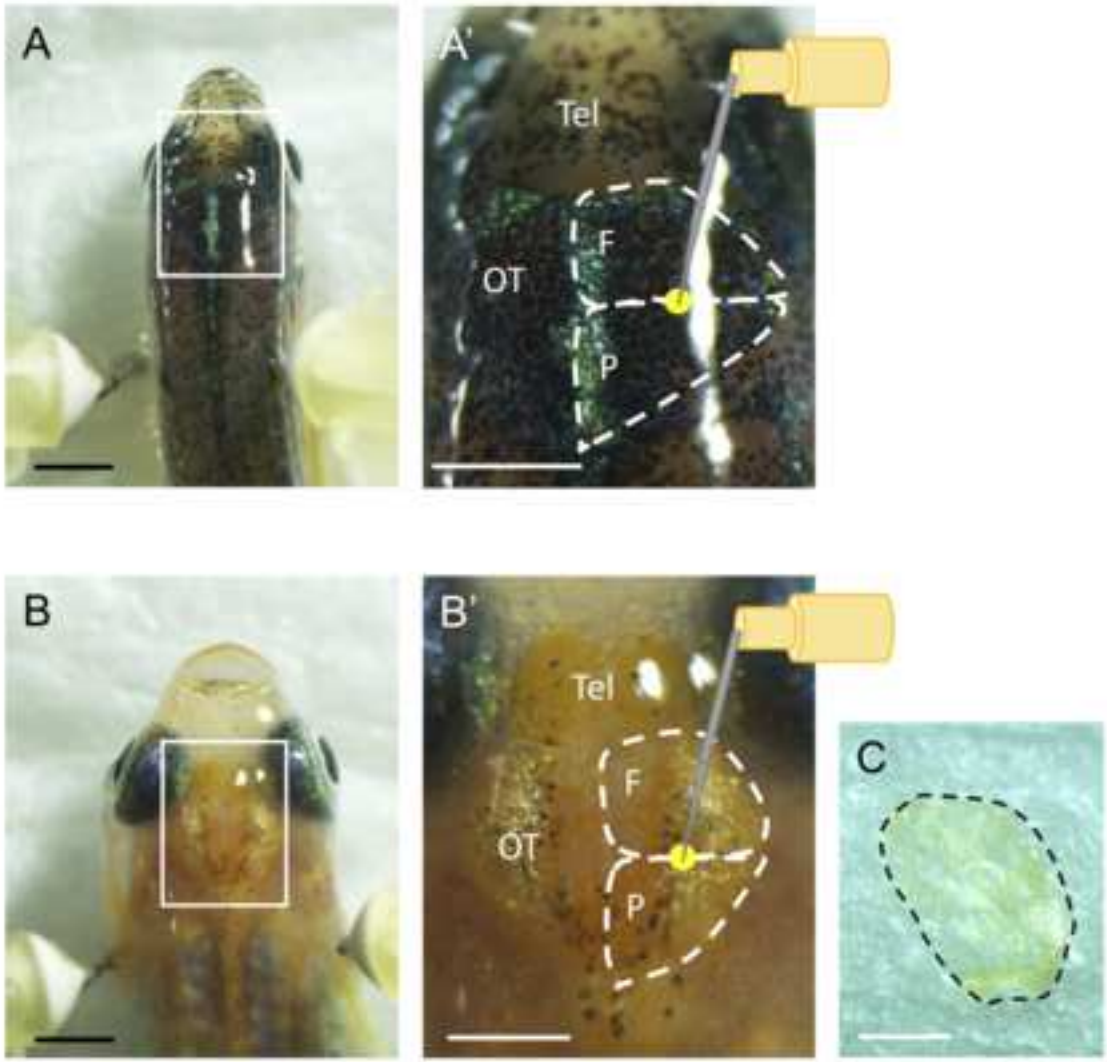

Figure 2

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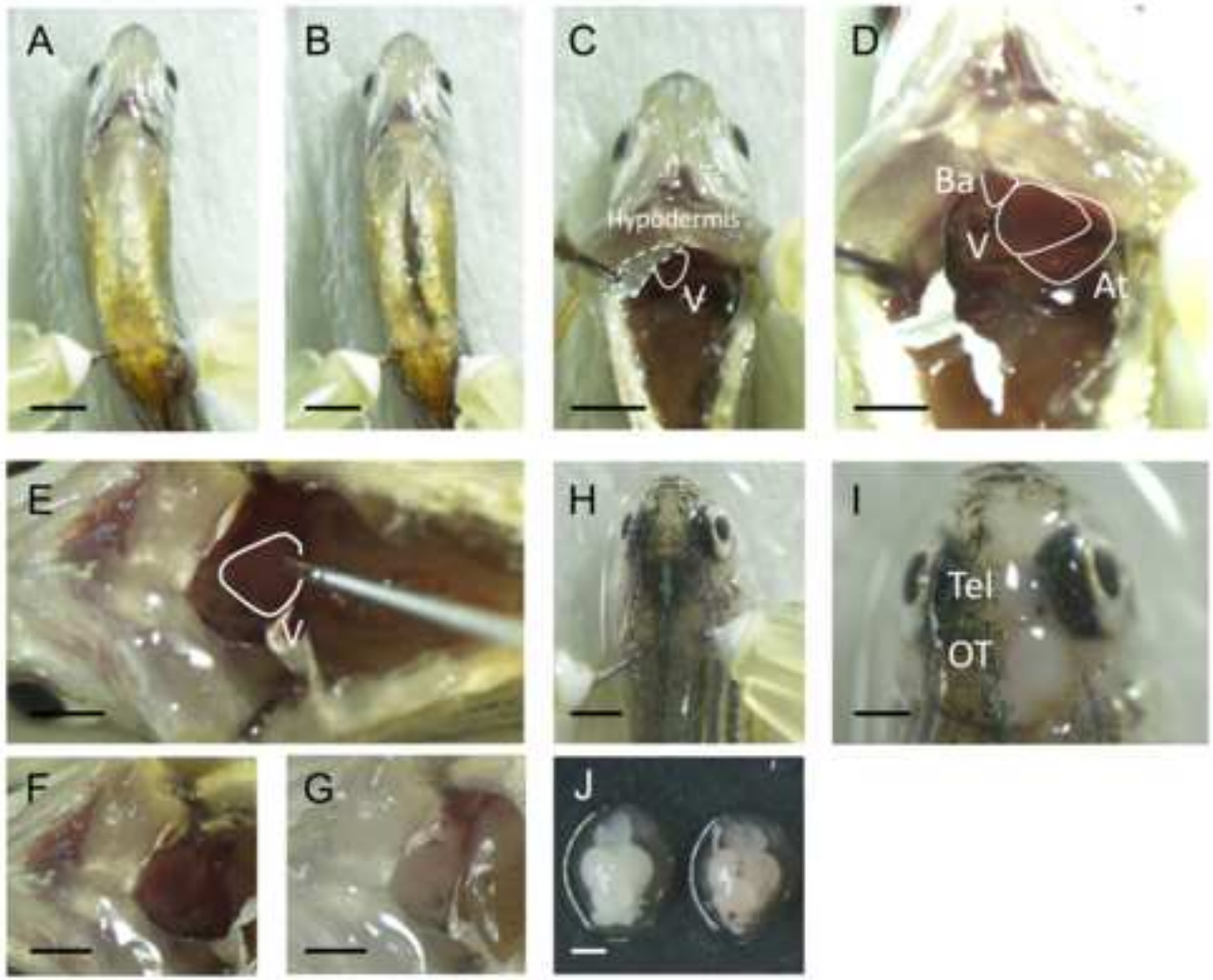

Figure 3

Click here to access/download;Figure;Figure 3.tiff

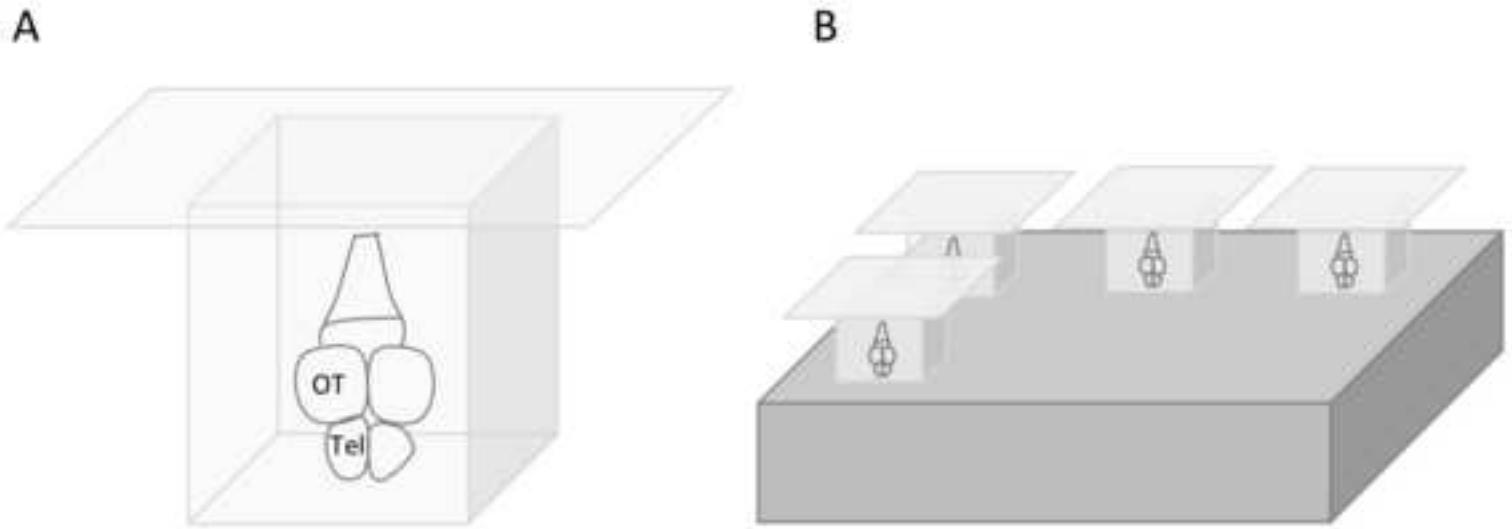

Figure 4

Click here to access/download;Figure;Figure 4.tiff

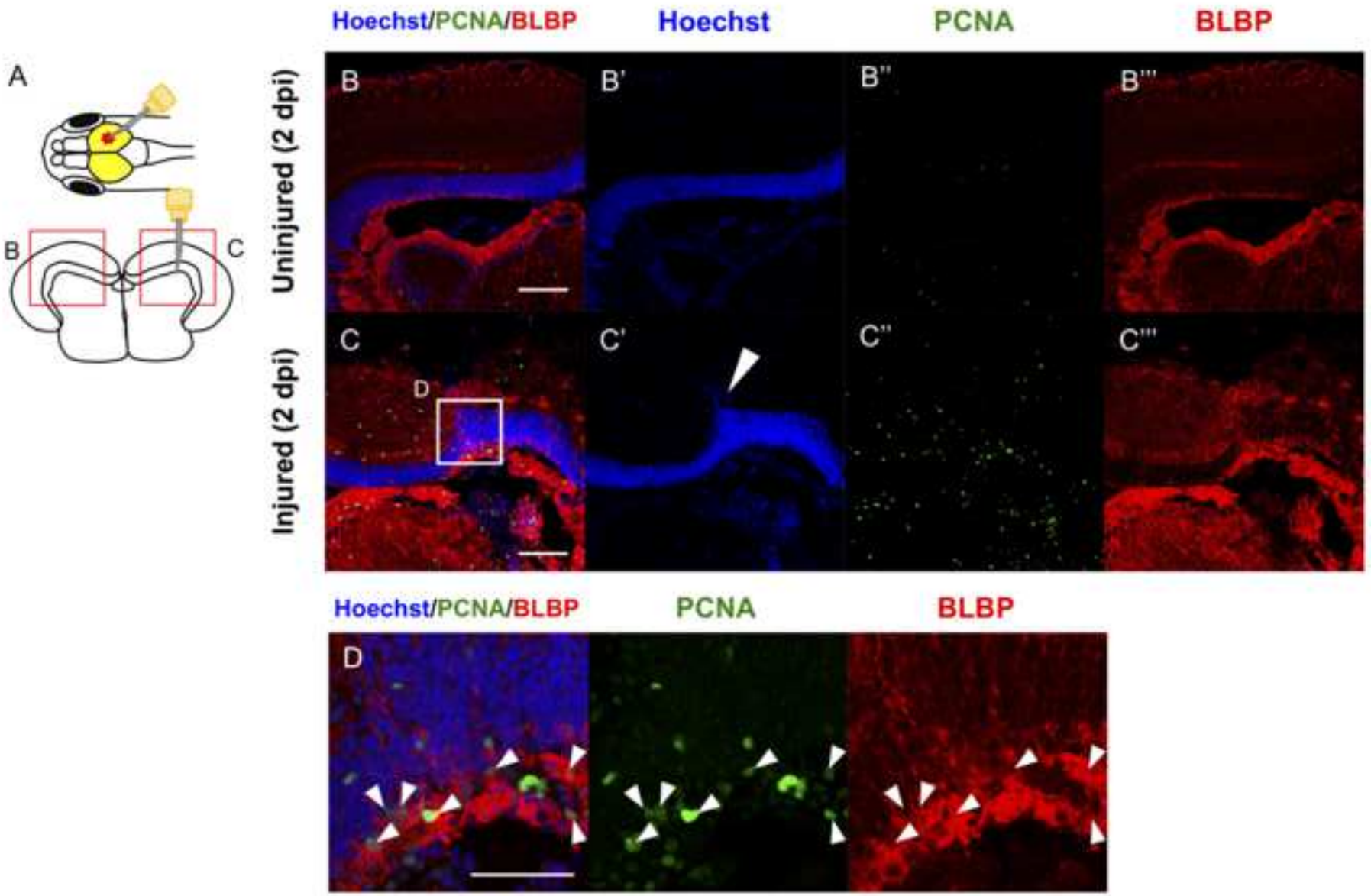

Figure 5

Click here to access/download;Figure;Figure 5.tiff

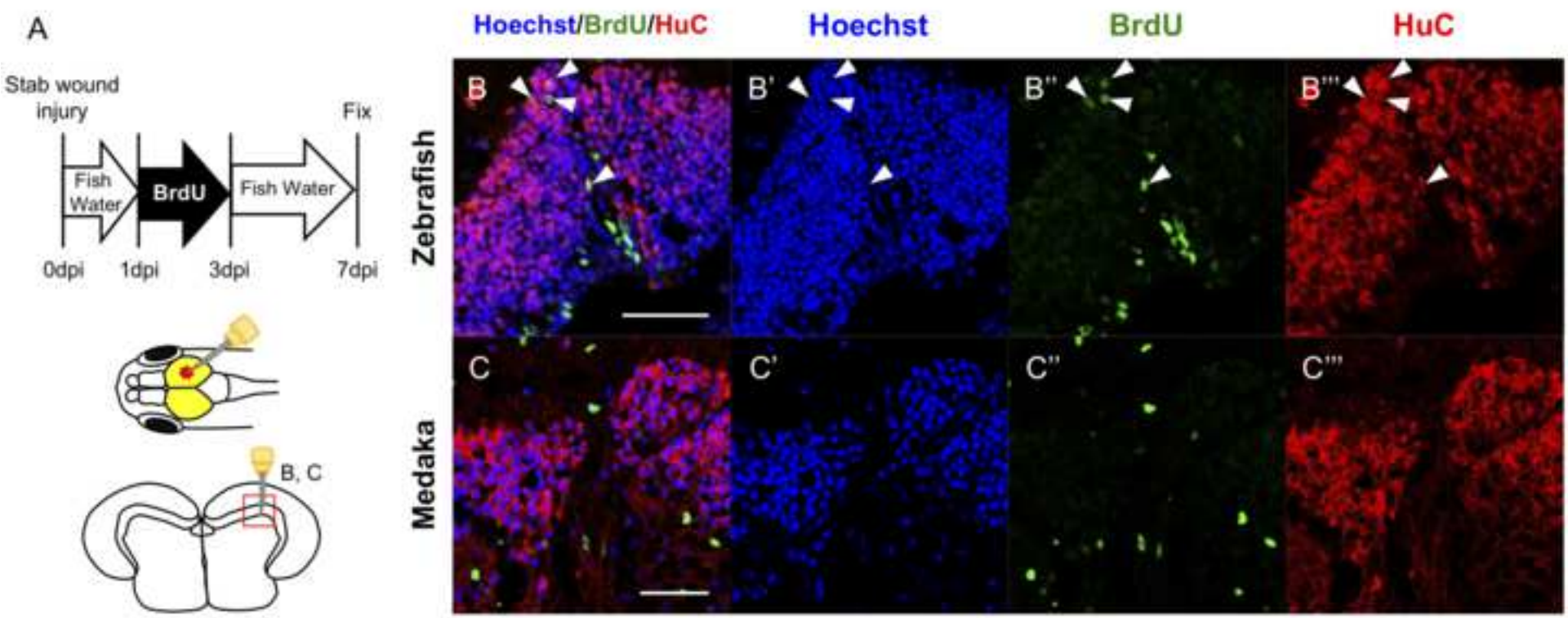

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Table of Materials
63166_R2_Table of Materials.xlsx

Rebuttal Letter
Click here to access/download;Rebuttal Letter;Rebuttal letter.docx

Sep. 27, 2021

Vidhya Iyer
Review Editor
Journal of Visualized Experiments

Dear Editor:

Thank you for the thoughtful and constructive feedback you provided regarding our manuscript, JoVE63166 "Stab wound injury model of the adult optic tecum using zebrafish and medaka for the comparative analysis of regenerative capacity".

We agree with editorial comments and reviewers' suggestions, and we have amended this by changing the contents as shown in red. The details of our responses are described in the following "Points to points response". We are certain that you will find this most recent version of our manuscript clears up the main issues that the editorial and the reviewers' comments indicated.

Thank you once again for your consideration of our paper. We look forward to hearing from you.

Sincerely,

Yuki Shimizu
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Points to points response

Editorial comments:

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. Please define all abbreviations at first use within the text and remove the list.

We have proofread the manuscript and defined all abbreviations and removed the list.

2. Please revise the following lines to avoid overlap with previously published work: 66-67, 283-284, 309-311, 317-319.

We have modified the sentences in Line 55-56, 280-281, 307-310, 315-317.

3. Please provide an email address for each author.

We have provided the email address for each author.

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

We have removed the personal pronouns.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have confirmed that the protocols contain everything that we would like show in the video.

6. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have used 12 points and highlighted the protocol text for inclusion in the protocol section of the video.

7. Please include a scale bar for ALL images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

We have confirmed that All images have scale bar.

8. As we are a methods journal, please ensure that the Discussion contains 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

We have confirmed that the Discussion contains these points as mentioned above.

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

We have confirmed that the references appear as mentioned above.

10. Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

We have sorted the Materials Table alphabetically by the name of the material.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes in detail a model of mechanical stab wound injury and the relevant procedure on how best to carry this out in small fish species including zebrafish and medaka. It is overall well written and while the described immunohistochemical procedures are now well standard, its inclusion in the procedure section does fit the title describing comparative analysis following the stab wound.

Overall a nice manuscript, well written and comprehensive.

The title clearly outlines the presented method and utility.

All relevant materials and equipment are listed in the table.

The protocol is detailed enough to allow anyone to reproduce the method in their own laboratory.

References are relevant and well covered.

Thank you for the reviewer's comments. We have revised the manuscript and figures as the reviewers pointed out.

Major Concerns:

The abstract states that this method is "robust and reproducible".

It would be nice if authors can show 3 examples of similar BrdU after stab injury OR a graph quantifying this to show the range of responses following such robust and reproducible injury.

Thank you for the reviewer's comments. We and other groups reported that this method induced reproducibly regenerative responses such as RGC proliferation in previous studies[13, 19-25]. We have added the following sentence in the Introduction. "This stab wound injury in the optic tectum is the robust and reproducible method[13,19-25]" (Line 62-63)

Minor Concerns:

The results and figures chosen are a good representation and useful for the technical aspect of the procedure. The fluorescent images are quite pixelated, but presumably this is only in this

submission draft.

Minor comments for the abstract:
- First word "although" should be replaced by "while".

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

- They state "novel" method, but stab injury has been used as shown in some of the referenced publications. Please clarify how this is different or novel, rather than a great description of a method used somewhat less defined perhaps.

Thank you for the reviewer's comment. We have removed "novel".

- End of the first paragraph, authors state "these" tissues, but they are only looking at the optic tectum. So perhaps "this tissue across these fish species" would be a more suitable description.

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

- As mentioned above, when describing this as robust and reproducible, please supply some results to showcase this. Otherwise perhaps describe that with the provided procedure, it will be more easy to generate comparable injuries and reduce any variability?

Thank you for the reviewer's comments. As described above, we and other groups reported that this method induced reproducibly regenerative responses such as RGC proliferation in previous studies[13-25].

Procedure section comments:

Important steps are overall listed and well explained in the procedure, there are a few points that could be clarified:

1.4 Describe the angle of the needles (vertical?) and refer to Figure 1A.
Thank you for the reviewer's comment. We have described the angle of the needles (vertical insertion) and referred to Fig. 1A.

1.5 Please describe how to "remove the scales", perhaps even as a separate step.
Is this done with forceps, scissors, scalpels, pushing with the 30G needle? If so, how (insert medial and push lateral?

Thank you for the reviewer's comment. We have described how to remove the scales using the 30 G needle (Line 104).

1.7 Replace the last word "necessary" with "appropriate".

2.1 tricaine is misspelt (missing "a").
Insert "a" before 10 mL syringe.

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

2.4 Describe needle direction (vertical?).

Thank you for the reviewer's comment. We have described the angle of the needles (vertical insertion).

2.5. insert two "the"s: "from THE anus to THE heart".

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

2.6 Very hard to see the "hypodermis, could this be labelled or outlined on the figure or zoomed in?

Thank you for the reviewer's comment. We have outlined the hypodermis on the figure to indicate it in Figure 2C.

2.9 needs full stop.

2.10 confirm if this is 4% para IN PBS?

3.3 delete "put" in the third line "and in that case the aluminion block should be….".

3.10 replace "completed" with "at".

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

4.1 Describe how to "dry" glass slides - flicking off excess solution, tipping slide and soaking up with paper towel?!?

Thank you for the reviewer's comment. We have added "within a slide rack" in line 195

4.2 Missing full stop at the end.

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

4.3. There is a formatting issue on the last line between the 37C for 30 min.
Perhaps this can be subdivided in PCNA antigen retrieval and BrdU antigen retrieval to make it shorter and easier to follow the two mutually exclusive?! Procedures.

Thank you for the reviewer's comment. We have divided the antigen retrieval into PCNA and BrdU section.

4.4 Describe how to "remove and wipe" - it could be mistaken to wipe over the sections.

Thank you for the reviewer's comment. We have replaced "Remove and wipe…" with "Absorb and remove remaining solution using paper towels".

4.10 To simplify, could be rephrased to "for 5 min at RT three times keeping the slides shaded."

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

4.13 Same comment regarding "remove and wipe".

Thank you for the reviewer's comment. We have replaced "Remove and wipe…" with "Absorb and remove remaining solution using paper towels".

4.14 Perhaps "observe AND IMAGE" could be added.

Thank you for the reviewer's comment. We have replaced as reviewer mentioned.

Reviewer #2:

Manuscript Summary:

"Stab wound injury model of the adult optic tectum using zebrafish and medaka for the comparative analysis of regenerative capacity" by Yuki Shimizu and Takashi Kawasaki introduces a stab wound injury model for the adult optic tectum of zebrafish and medaka. This method is available for small teleosts and enables searchers to compare their regenerative capacity and investigate novel molecular mechanisms.

Thank you for the reviewer's comments. We have revised the manuscript and figures as the reviewers pointed out.

Major Concerns:

1. Page9, lines 332-334: "The precise location and depth of insertion helps to create reproducible injuries in experiments; this paper provides clear guides for creating similar injuries each time." However, there are no more details to describe in step 1.5. Such as, how deep should the needle insertion into the optic tectum once it through the skull, should the beveled of the needle tip be subtracted? Are zebrafish and medaka share the same parameters?

Thank you for the reviewer's comment. We have added the following sentences in step 1.5.

"The insertion depth is approximately 0.75 mm, almost equal to half the length of the 30 G bevel. This insertion depth similarly induces the brain injury on zebrafish and medaka because of similar body size of these fish."

2. Should a sham group be performed in this injury model, especially in those studies that want to compare gene expression differences?

Thank you for the reviewer's comment. We confirmed that the sham operation has seemingly no histological effect such as induction of RGC proliferation, though the sham operation may be important especially when analyzing gene expression changes, as the reviewers mentioned.

Minor Concerns:

1. The word "optic tecum" in the title (page 2, line 3) should be "optic tectum". The same problem exists on page 1 and in line 29 and line 273.

Thank you for the reviewer's comments. We have corrected as reviewer mentioned.

2. Since the hole in the skull is not sealed after the stab, what is the survival rate after step 1.6?

Thank you for the reviewer's comments. The stab wound injury without seal showed enough high survival rate in the case of not only optic tectum but also telencephalon.

3. Does it needs to perfuse PFA through the ventricle during intracardial perfusion at step 2.8.

Thank you for the reviewer's comments. We confirmed that it is enough to fix a dissected brain with 4 % PFA because the zebrafish brain is small. The purpose of PBS perfusion is to remove blood.

4. In general, tissue dehydration in sucrose is carried out in gradient concentrations. Is it necessary to add a middle concentration in step 3.2?

Thank you for the reviewer's comments. Generally, in the case of zebrafish tissue such as heart and brain, one step dehydration with 30 % sucrose is enough.

5. In step 3.5, is there any trick to ensure that the coronal section coincides with the lesion channel. Once the cutting plane is tilted, what effect will it have on the result analysis?

Thank you for the reviewer's comments. During cutting telencephalon, the orientation is adjusted. We have added the following sentence "Note: Adjust the orientation of the cutting plane during cutting the telencephalon." (line 185-186).

6. Except for the nuclear staining that could be used to confirm the injury site (line 273), are there any other dyes that could clearly show the trajectory of the stab injury?

Thank you for the reviewer's comment. Hematoxylin and Eosin staining is also available, and we have added it in the sentence (line 269).

7. Abbreviation should add os frontale (F) and os parietale (P)?

Thank you for the reviewer's comment. We have added these abbreviations.

8. Recommended adding two articles in references.
URL: http://www.jove.com/video/51753 and URL: http://www.jove.com/video/50987

Thank you for the reviewer's comment. We have already cited the first articles. The second one is related to optic nerve regeneration not to brain regeneration.

Reviewer #3:
Manuscript Summary:
The manuscript is well written and easy to understand.

Thank you for the reviewer's comments. We have revised the manuscript and figures as the reviewer pointed out.

Major Concerns:
The method is not novel and already described (although not in this detail) in the previous publication in Front Cell Dev Biol., which probably is ok for a Methods Collection. However the left part of Fig 4 and 5 is similar to parts of the figures in the previous publication. I don't know if this may pose some copyright issues.

Thank you for the reviewer's comments.
Front Cell Dev Biol. indicates that "All Frontiers articles from July 2012 onwards are published with open access under the CC-BY Creative Commons attribution license (the current version is CC-BY, version 4.0). This means that the author(s) retains copyright, but the content is free to download, distribute, and adapt for commercial or non-commercial purposes, given appropriate attribution to the original article." We cited our publication in Front Cell Dev Biol related to figures in this manuscript.

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