OXFORD

BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2024, **9(1),** bpae073

https://doi.org/10.1093/biomethods/bpae073
Advance Access Publication Date: 10 October 2024
Protocols

A reproducible method to study traumatic injury-induced zebrafish brain regeneration

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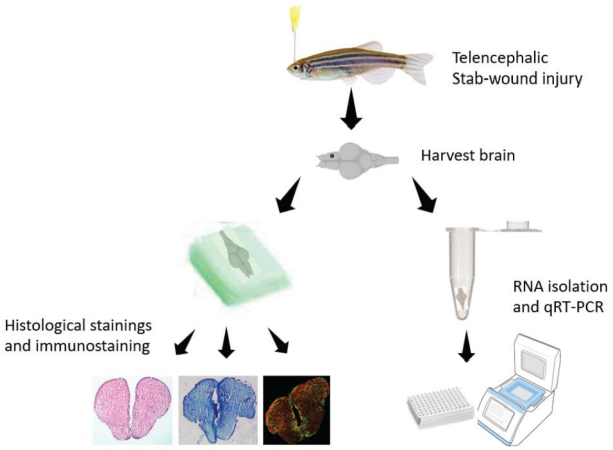
Abstract

Traumatic brain injury (TBI) can be caused by a sudden blow or jolt to the head, causing irreversible brain damage leading to cellular and functional loss. Mammals cannot repair such damage, which may increase the risk of progressive neurodegeneration. Unlike mammals, lower vertebrates such as zebrafish have the astounding capability to regenerate their brains. A model system would be of great value to study zebrafish brain regeneration. Here, we describe a physical method to induce traumatic injury in the zebrafish brain and outline a pipeline to utilize this model system to explore various aspects of brain regeneration. This will significantly advance the fields of regenerative biology and neuroscience. The method includes inducing TBI and validating this through histological assays, immunohistochemistry, and gene expression analysis. By using this model system, researchers will be able to gain valuable insights into the cellular and molecular mechanisms underlying brain regeneration. Understanding these mechanisms could lead to the identification of potential strategies to address neurodegenerative conditions in higher vertebrates.

Keywords: zebrafish; brain; regeneration; histology; immunohistochemistry

Graphical abstract



Introduction

The brain is an essential part of the central nervous system controlling all the body's necessary functions, such as memory, motor activities, breathing, touch, hunger, etc., and any injury to the brain therefore leads to an impact on one or more of these processes. Over the course of evolution, mammals have largely lost the capacity to regenerate a significantly injured brain, leading to various neurological disorders and defects upon such injury. By contrast, lower vertebrates such as zebrafish possess remarkable brain regenerative capacity due to the presence of

Received: 6 August 2024. **Revised:** 13 September 2024. **Editorial decision:** 13 September 2024.

approximately sixteen neurogenic niches across the brain [1–4]. A stab-wound injury model has been widely established to study zebrafish brain regeneration, which involves induction of injury in the parenchymal region (which contains mostly post-mitotic neurons) of the telencephalon [5–7].

The zebrafish brain telencephalon is the most studied and contains progenitors along the outer surface of the ventricular zone, which are mostly quiescent and very few of these give rise to new neurons under physiological conditions [8, 9]. These new-born neurons are deposited in the periventricular region which is a one-to-two-cell layer below the neurogenic layer of cells [1, 2, 10]. Upon injury-induced regeneration in zebrafish brain, various events occur at the cellular and molecular level. Immediately after the injury, microglia (the resident macrophages in the brain) are activated in response to the signals from injury-induced inflammation and cell death [11]. This is followed by accumulation of a progenitor cell population at the site of injury where these give rise to new-born neurons, thereby regenerating the lost tissue. These newly born neurons are efficiently incorporated into the neural circuitry [4, 12, 13]. This process involves an intricate interplay of various cellular processes and signalling pathways which are essential to study for a deeper understanding of the regeneration process [14].

Here, we outline a systematic approach to use this model system to study diverse characteristics of brain regeneration in zebrafish which offers promises for advancing research in mammalian brain regeneration studies. In this approach, we describe a method wherein immunohistochemistry and quantitative real-time PCR can be used to study spatial and temporal changes at molecular, cellular, and gene levels using various established markers.

Materials and reagents

Biological materials

1. Zebrafish strain AB (4 to 8 months old)

Reagents

1. Ethyl 3-aminobenzoate (methanesulfonate) (Cayman, catalog number: 20660)
2. Paraformaldehyde (Sigma, catalog number: 158127)
3. Sodium azide (Himedia, catalog number: MB075)
4. Sodium chloride (NaCl) (Himedia, catalog number: MB023)
5. Sodium phosphate dibasic (SRL, catalog number: 45932)
6. Sodium phosphate monobasic (SRL, catalog number: 59443)
7. Trizma base (Sigma-Aldrich, catalog number: T6066)
8. Sodium hydroxide pellets (SRL, catalog number: 68151)
9. Hydrochloric acid (Amplura, catalog number: 1.93401.0521)
10. 5-Bromo-2-Deoxyuridine extrapure, 98% (BrdU) (SRL, catalog number: 57418)
11. Mowiol® 4-88 (Sigma-Aldrich, catalog number: 81381)
12. 1,4 Diazabicyclo(2,2,2) octane (DABCO) (CDH, catalog number: CAS# 280_57_9)
13. Tri-sodium citrate dihydrate (CDH, catalog number: 30128)
14. Glycerol (Amplura, catalog number: 1.94501.0521)
15. Normal goat serum (Elabsciences, catalog number: E-IR-R111)
16. Ethanol (Emsure, catalog number: 1.00983.0511)
17. Haematoxylin (SRL, catalog number: 48441)
18. Eosin yellow (SRL, catalog number: 29391)
19. Acetone (Merck, catalog number: 1.07021.0521)
20. Glacial acetic acid (SRL, catalog number: 90868)
21. DPX mounting medium (SRL, catalog number: 88147)
22. Paraffin wax (SRL, catalog number: 47994)
23. Xylene (SRL, catalog number: 89159)
24. iTaq Universal SYBR Green Supermix (Biorad, catalog number: 1725121)
25. Hard shell PCR plates (Biorad, catalog number: HSP9601)
26. Plate sealers (Biorad, catalog number: MSB1000)
27. Trizol (Invitrogen, catalog number: 15596026)
28. Chloroform (Merck, catalog number: 1.94506.0521)
29. Isopropanol (Sigma, catalog number: I9516)
30. Nuclease-free water (Qiagen, catalog number: 129115)
31. RNeasy Lipid Tissue Mini Kit (Qiagen, catalog number: 1023539)
32. Real-time primers (Eurofin)
33. BrdU antibody (DSHB, catalog number: DSHB-S1-862)
34. PCNA antibody (Santa Cruz, catalog number: sc25280)
35. GFAP antibody (Abcam, catalog number: ab154474)

Solutions

1. 10x PBS buffer (See recipes)
2. Tricaine (See recipes)
3. 4% Paraformaldehyde (See recipes)
4. 5 mM BrdU solution (See recipes)
5. 10 mM sodium citrate buffer (See recipes)
6. Mowiol mounting medium (See recipes)
7. 70% Ethanol (See recipes)
8. 80% Ethanol (See recipes)
9. 95% Ethanol (See recipes)
10. Ethanol: Xylene (1:1)
11. Alcohol acid (See recipes)
12. Eosin solution (See recipes)
13. 10x TBE (See recipes)

Recipes

1. Tris-HCl (1M, pH 9.0)—Needed for making Tricaine stock solution

Reagent	Amount
Tris base	12.114 gm
Total volume	100 ml

   a) Weigh Tris base and dissolve in 80 ml Milli-Q.
   b) Maintain the pH to 9.0 and make up the total volume to 100 ml.

2. Tris-HCl (1M, pH 8.5)—Needed for making Mowiol mounting medium

Reagent	Amount
Tris base	12.114 gm
Total volume	100 ml

   a) Weigh Tris base and dissolve in 80 ml Milli-Q.
   b) Maintain the pH to 8.5 and make up the total volume to 100 ml.

3. Tricaine stock solution

Reagent	Amount
Tricaine powder	400 mg
Tris-HCl (1 M, pH 9.0)	2 ml
Milli-Q water	97.9 ml
Total volume	100 ml

a) Maintain the pH to 7.4 before making up the total volume to 100 ml.
b) Prepare aliquots and store the tricaine stock solution in −20°C.
c) To make anesthetizing solution, add 3 ml of tricaine stock solution in 30 ml system water.

4. 10X Phosphate-buffered saline

Reagent	Amount
Na_2HPO_4	10.9 gm
NaH_2PO_4	3.2 gm
NaCl	87.66 gm
Total volume	1000 ml

a) Weigh and add the salts mentioned above in a 1 litre screw-cap bottle.
b) Add 800 ml Milli-Q and mix with a magnetic stirrer.
c) Maintain the pH to 7.4 and make up the total volume to 1000 ml.
d) Add 100 ml of this 10x PBS and add 900 ml Milli-Q to make 1 litre of 1x working PBS buffer.

5. Four percent of paraformaldehyde solution

Reagent	Amount
Paraformaldehyde powder	4 gm
10x PBS	10 ml
1M NaOH	1 ml
Total volume	100 ml

a) Weigh 4 gm paraformaldehyde powder and add 50 ml Milli-Q and 1 ml NaOH (1M).
b) Mix with stirring at 60°C until paraformaldehyde is dissolved completely.
c) Add 10 ml PBS (10x) and let it to cool down to room temperature.
d) Maintain the pH to 7.4 and make up the volume to 100 ml with Milli-Q.
e) Make 10 ml aliquots and store at −20°C.
f) Thaw it at RT before use.

6. BrdU solution

Reagent	Amount
BrdU powder	368.6 mg
Total volume	20 ml

a) Add the given amount of BrdU powder in a 50-ml centrifuge tube.
b) Dissolve it in 20 ml Milli-Q to make 30 mM stock solution.
c) Make 4 ml aliquots of 30 mM and store at −20°C.
d) To make 5 mM working BrdU solution, thaw one 4 ml aliquot and add 20 ml system water.

7. 10 mM Sodium citrate buffer

Reagent	Amount
Tri-sodium citrate dihydrate	880 mg
Total volume	300 ml

a) Add tri-sodium citrate dihydrate to a 500-ml screw-cap bottle.
b) Dissolve it in 250 ml Milli-Q
c) Maintain the pH to 6.0 and make up the total volume to 300 ml.

8. Mowiol mounting medium

Reagent	Amount
Mowiol 4-88	2.4 gm
Glycerol	5 ml
Milli-Q	6 ml
Tris-HCl (0.2M, pH 8.5)	12 ml
DABCO	23 mg
Total volume	23 ml

a) Add Mowiol 4-88 to a 50-ml centrifuge tube.
b) Add glycerol, Milli-Q, and Tris-HCl (0.2M, pH 8.5). Mix it overnight at 50°C by stirring.
c) Add DABCO and mix at 50°C for 5 min.
d) Make 1 ml aliquots and store at −20°C.

9. Eosin solution

Reagent	Amount
Eosin	125 mg
70% ethanol (v/v)	50 ml
Acetone	Few drops
Total volume	50 ml

10. Alcohol acid

Reagent	Amount
Hydrochloric acid	0.5 ml
Absolute ethanol	35 ml
Double-distilled water	14.5 ml
Total volume	50 ml

11. 70% Ethanol

Reagent	Amount
Absolute ethanol	35 ml
Double distilled water	15 ml
Total volume	50 ml

12. 80% Ethanol

Reagent	Amount
Absolute ethanol	40 ml
Double-distilled water	10 ml
Total volume	50 ml

13. 95% Ethanol

Reagent	Amount
Absolute ethanol	47.5 ml
Double-distilled water	2.5 ml
Total volume	50 ml

14. 10x TBE

Reagent	Amount
Tris base	121.1 gm
Boric acid	68.1 gm
EDTA	7.4 gm
Total volume	1000 ml

a) Weigh and add the salts mentioned above in a 1-litre screw-cap bottle.
b) Add 800 ml Milli-Q and mix with a magnetic stirrer.
c) Once dissolved, make up the total volume to 1000 ml.
d) Add 100 ml of this 10x TBE and add 900 ml Milli-Q to make 1 litre of 1x working TBE buffer.

Laboratory supplies

1. 30G needles (BD Microlance, 30G ½", 0.3 x 13 mm, catalog number: 304000)
2. 50-ml centrifuge tube (Tarsons, catalog number: 546041)
3. 15-ml centrifuge tube (Tarsons, catalog number: 546021)
4. 1.5-ml microcentrifuge tube (Thermo Fisher, catalog number: 509-GRD-Q)
5. Forceps (Surtex Instruments, catalog number: FR-780-10)
6. 100-ml beaker (Tarsons, catalog number: 421020)
7. Tissue embedding cassettes (Medimeas)
8. Embedding moulds (15 mm X 15 mm X 6 mm) (Medimeas)
9. Kimtech Science™ Kimwipes® (Kimberly-clark, catalog number: 34120)
10. Paper/thermocol cutter
11. Aluminium foil
12. Humidifying chamber (slide storage box with bottom covered with aluminium foil and filled with double distilled water up to 1/4th height of the box)
13. Slide storage box (PolyLab, catalog number: 62106)
14. Coverslips (Blue Star 22 mm X 60 mm)
15. Positively charged glass slides (Fisher scientific, catalog number: 1255015)
16. Milli-Q water
17. Tap water
18. 90 mm Petri-plate (Tarsons, catalog number: 460091)
19. Sponge
20. Thermocol piece covered with aluminium foil
21. Liquid nitrogen
22. Double-distilled water
23. Pencil
24. Hydrophobic pen (PAP pen) (Sigma-Aldrich, catalog number: Z672548-1EA)
25. Coplin jars (PolyLab, catalog number: 61101)
26. Maxipense maxiamp PCR® tubes (Tarsons, catalog number: 510075)

Equipment

1. pH meter (Jaisbo, catalog number: 5108)
2. Magnetic stirrer with hot plate (Jaisbo, catalog number: 5054/1)
3. Dissection microscope (Debro DSZ-55)
4. Slide warming table (Medimeas M-SWT)
5. Microtome (Medimeas MRM-RM)
6. Tissue floatation bath (Medimeas, catalog number: M-TFB)
7. Rotor with 50-ml centrifuge tube attachment (Jaisbo, catalog number: 5075/4)
8. Gel rocker (Jaisbo, catalog number: 5078)
9. Paraffin wax bath (Medimeas, catalog number: M-ATP-WB1)
10. Confocal microscope (Leica TCS SP8)
11. Bright-field microscope (Nikon eclipse TS2)
12. Microwave oven (Samsung MS23K3513AK/TL)
13. Nanodrop™One/One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Scientific, catalog number: ND-ONE-W
14. Homogenizer (Jaisbo, catalog number: MT-13K)
15. CFX96 Touch Deep Well™ Real-Time PCR Detection System (Biorad, catalog number: 1854096)

Software and data sets

1. Fiji: Imagej (version: 2.9.0, date: September 14, 2022)
2. BioRad CFX maestro 1.1 (Version: 4.1.2433.1219, date: December, 2021)

Methods

TBI

TBI in the zebrafish was conducted as described in Kishimoto et al. [6] and Schmidt et al. [5]. The zebrafish were anesthetized in diluted MS-222 (3-amino benzoic acid ethyl ester, also called ethyl 3-aminobenzoate) which is commonly called Tricaine, and is prepared by adding 5 ml Tricaine stock solution (see recipe in Materials and Reagents) and making up the volume to 30 ml with tank water (in which zebrafish are maintained) until they are completely still (45–60 sec). A telencephalic lesion was introduced by poking the telencephalic hemisphere of adult fish that were about 4–6 months old with a body measurement of 25–32 mm long using a 30 g (outer diameter 300 µm) needle. Essentially, a 30 g needle was inserted vertically into the medial telencephalic hemisphere of one of the telencephalic hemispheres with the least force possible. The needle is gently inserted through the skull to induce an injury precisely in the central dorsal telencephalic region while maintaining perpendicular orientation with regard to the telencephalon. This process is facilitated by the fact that the telencephalic hemispheres can be seen through the skull. It is ensured that the lesion introduced is no more than 2 mm deep and only a single poke/lesion is introduced. If done gently, this process never leads to any damage to the brain in general except the intentionally induced injury. The fish were then returned to their respective tanks to recover after the lesion is introduced and sacrificed at the desired time points, as explained below.

BrdU labelling

BrdU (5-bromo-2-deoxyuridine) is commonly used as an S-phase cell cycle marker. BrdU solution (5 mM) was prepared from a 30 mM stock solution. The working solution can be stored at −20°C and should be brought to room temperature before use. 24 ml of 5 mM BrdU solution was taken in a 100-ml beaker, and zebrafish was immersed in it for 4 hrs prior to the sacrifice.

Harvesting the brain

Post-BrdU labelling, the fish was anesthetized in tricaine until any movement in the gills and operculum stopped. 4% (w/v) paraformaldehyde (PAF) solution should be thawed beforehand and kept on ice during the dissection. Each fish was carefully placed on a thermocol stage or a wet sponge and oriented under the microscope, as shown in Fig. 2 (A2). Using sharp forceps, a

horizontal cut just below the head was made and the skull was carefully removed in small pieces. Then both the eyes were removed by carefully holding the optic nerves using forceps through the eye socket and cutting them using the forceps. Once the brain was completely exposed, it was carefully harvested using the forceps and placed in a clean petri-dish containing 1x PBS (phosphate buffered saline, see recipe in the Materials and reagents section). The brain was fixed in 4% (w/v) paraformaldehyde in a 15-ml Falcon tube (filled with 8–10 ml 4% (w/v) paraformaldehyde) at 4°C on a gel rocker for 2–4 hrs. The brain can also be transferred into a MCT (microcentrifuge tube) after the dissection and processed for RNA isolation immediately. Alternatively, the brain can be transferred into an MCT, snap-frozen in liquid nitrogen, and stored at −80°C (for up to 3–4 months) for either protein extraction or RNA isolation.

Tissue preparation for paraffin block

Post-fixation, the brain was washed with 1x PBS three times, 5 min each. At this point, the brain can be stored in 4°C in 1x PBS-containing sodium azide (1 gm/l) for up to a month or can be immediately processed for making paraffin block. When the brain is stored in 1x PBS with sodium azide, make sure to wash the brain with 1x PBS 3–4 times, 10 min each, in order to remove any traces of sodium azide. Once the brain is washed with 1x PBS, carry out the following steps (Table 1) in a 50-ml Falcon tube on rotation. Note: The 50-ml Falcon tube should be filled with at least 30 ml of the solutions mentioned in the Table 1.

For coronal sections of the telencephalon, a small piece of the block containing only the brain is cut (from the paraffin block prepared as mentioned above) using a sharp cutter and placed in the mould with the telencephalon facing towards the mould. Paraffin wax was poured halfway through the mould, and the labelled cassette was placed on the top (lid removed), filled with paraffin wax (Fig. 2). This set-up was then placed in the fridge for the wax to set. The moulds were removed the next day. The prepared blocks can be stored in the fridge until sectioned.

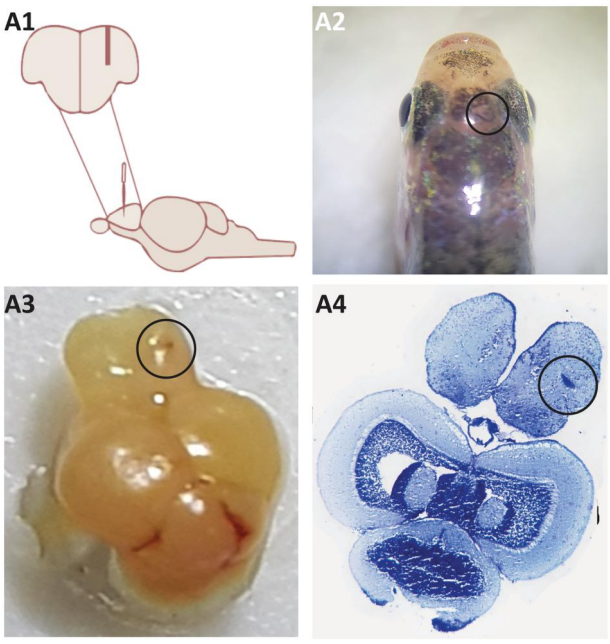


Figure 1. Telencephalic stab-wound injury model of zebrafish brain regeneration. (A) Illustration of stab-wound injury. A2 and A3 show the injury in the right hemisphere (encircled in black). A4 shows toluidine blue O-stained zebrafish brain section in transverse orientation (injury encircled in black).

Sectioning

Blocks should be taken to the microtome on ice. The blade should be carefully placed in the blade holder and set the block in the microtome. The tissue floater should be set at 37°C. Five-micrometre-thick sections were cut, and using forceps or a 200-µl tip the sections (ribbon of 4–5 sections) were put carefully in the tissue floater. The sections were collected on the positively charged or coated glass slides and dried overnight. The slides should be labelled with a pencil and stored at 4°C.

HE (haematoxylin and eosin) staining

The slides were heated at 60°C for 10 min, and dewaxing was carried out in xylene.

1. Xylene I—1 min
2. Xylene II—1 min
3. Xylene III—2 min

The slides were then rehydrated in an ethanol gradient

1. 100% ethanol—plunged 10 times
2. 100% ethanol—plunged 10 times
3. 95% (v/v) ethanol—plunged 10 times
4. 80% (v/v) ethanol—plunged 10 times
5. 70% (v/v) ethanol—plunged 10 times
6. Double-distilled water—2 min

The slides were then immersed in commercially available undiluted haematoxylin (filtered before use) for 7–8 min, followed by washing in tap water for 2 min and plunging in alcohol acid (see recipe in the Materials and reagents section) 9 times. The slides were washed in tap water for 10 min and immersed in 0.25% (w/v) eosin aqueous solution for 90 sec. The slides were dehydrated in an ethanol gradient.

1. 70% (v/v) ethanol—plunged 5 times
2. 80% (v/v) ethanol—plunged 5 times
3. 95% (v/v) ethanol—plunged 10 times
4. 100% ethanol—plunged 10 times
5. 100% ethanol—plunged 10 times
6. Xylene 1—plunged 10 times
7. Xylene 2—plunged 10 times
8. Xylene 3—plunged 10 times

The slides were carefully wiped with lint-free tissue without touching the sections and were mounted with DPX (dibutylphthalate polystyrene xylene) mounting medium. The slides should be placed undisturbed at room temperature for air-drying overnight. The slides were then visualized under a microscope.

Toluidine blue O staining

The slides were heated at 60°C for 10 min and dewaxing was carried out in xylene.

1. Xylene I—1 min
2. Xylene II—1 min
3. Xylene III—2 min

The slides were then rehydrated in ethanol gradient

1. 100% ethanol—plunged 10 times
2. 100% ethanol—plunged 10 times

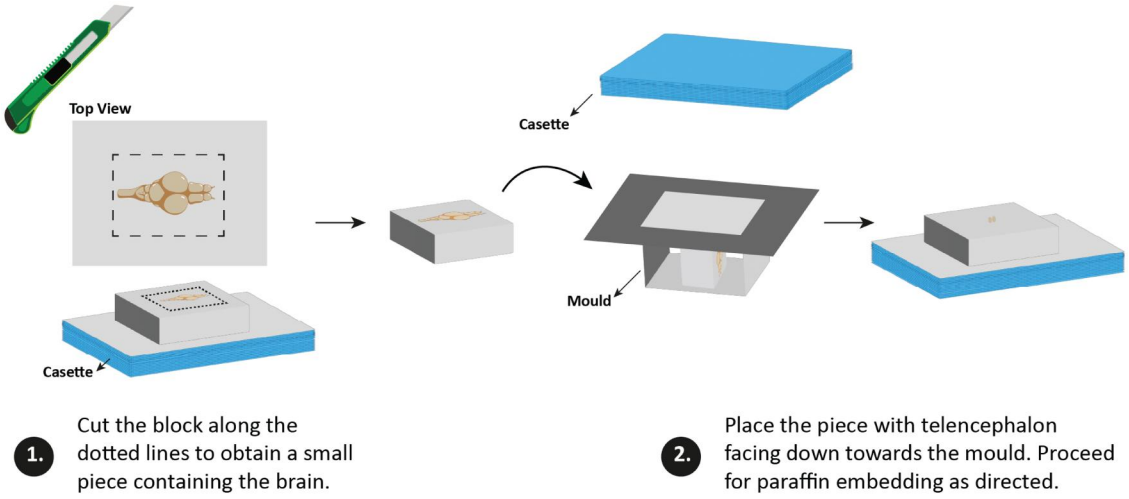


Figure 2. An illustration showing the preparation of the paraffin block of zebrafish brain in the coronal orientation. The paraffin block is initially prepared with the brain in transverse orientation. Once the block is completely set in the fridge, it is cut as shown in step 1 to obtain only a small piece of the paraffin block containing the brain. The block is then placed in the mould as shown in step 2 and set in fridge to have the paraffin block of the brain in the desired orientation to obtain coronal sections.

Table 1. Tissue processing steps for paraffin block preparation.

Solution	Time	Temperature
80% (v/v) ethanol	20 min	RT
80% (v/v) ethanol	20 min	RT
95% (v/v) ethanol	20 min	RT
95% (v/v) ethanol	20 min	RT
100% ethanol	45 min	RT
100% ethanol	45 min	RT
100%ethanol+xylene (50/50 v/v)	25 min	RT
100%ethanol+xylene (50/50 v/v)	25 min	RT
Xylene 1	25 min	RT
Xylene 2	25 min	RT
Paraffin 1	2 hrs	60°C
Paraffin 2	2 hrs	60°C

Note: At the 'Xylene 2' step, switch on the paraffin wax bath (see equipment in Materials and Reagents section) at 60°C and fill it with paraffin wax up to a height of 4–5 cm (when melted). For the 'Paraffin 1 and 2' steps, the harvested brain from 'Xylene 2' was placed in a labelled cassette using forceps and the cassette was immersed in the wax bath (containing melted paraffin wax). The wax bath (ON at 60°C) was then kept on a gel rocker in motion. After the 'Paraffin 2' step, the processed brain tissue was carefully placed in the mould (with wax filled halfway through the mould) in a transverse orientation. The labelled cassette was placed on the top (lid removed) and filled with paraffin wax. This set-up was then placed in the fridge for the wax to set. While placing the brain in the transverse section, remember which direction the telencephalon is (mark it on the cassette itself). This will be necessary while making coronal sections.

3. 95% (v/v) ethanol—plunged 10 times
4. 80% (v/v) ethanol–plunged 10 times
5. 70% (v/v) ethanol—plunged 10 times
6. Double distilled water—2 min

The sections were incubated with 1% (w/v) toluidine blue O solution made in double-distilled water for 30–45 sec, and the slides were immediately washed twice (10 min each) with tap water. The slides were then dried at room temperature or on a hot plate (set at 30°C). The slides were mounted with DPX mounting medium. The slides were placed undisturbed at room temperature for air-drying overnight and were visualized under a microscope.

Immunohistochemistry

The slides were heated at 60°C for 10 min, and dewaxing was carried out in xylene.

1. Xylene I—1 min
2. Xylene II—1 min
3. Xylene III—2 min
4. Xylene IV—overnight (on gel rocker)

The slides were rehydrated in ethanol gradient.

1. 100% ethanol—plunged 10 times
2. 100% ethanol—plunged 10 times
3. 95% (v/v) ethanol—plunged 10 times
4. 80% (v/v) ethanol—plunged 10 times
5. 70% (v/v) ethanol—plunged 10 times
6. Double distilled water—2 min
7. 1x PBS wash—5 min

Freshly prepared 0.01 M sodium citrate buffer (pH 6.0) was used for antigen retrieval. The slides were immersed in boiling citrate buffer twice, 5 min each. The slides were then (still in buffer) cooled in the fridge for 30 min followed by washing with 1x PBS for 5 min, and the sections were blocked in 1x normal goat serum (NGS) for 30 min at room temperature. Normal goat serum is available commercially as 1x and 10x. 1x NGS can be used directly, whereas 10x NGS is diluted with autoclaved Milli-Q water to 1x before use. NGS was then removed by carefully dabbing it with a lint-free tissue without touching the sections followed by outlining the sections using a hydrophobic pen. This ensures that a small volume of antibody dilution is sufficient, and simultaneously, different antibodies can be used on different sections on the same slide. The slides were placed in a humidified chamber (See lab supplies section in Materials and Reagents). Primary antibody dilutions were made in 1x NGS, and the sections were incubated with it at 4°C overnight. Antibodies used here: for proliferating markers, anti-BrdU (1:50) and anti-PCNA (proliferating cell nuclear antigen) (1:150). As a glial cell-specific marker,

anti-GFAP (glial fibrillary acidic protein) (1:500) was used. The slides were then washed with 1x PBS twice, 5 min each. Secondary antibody incubation was carried out for 1 hr at room temperature (in the dark). The slides were washed with 1x PBS twice, 5 min each, and the sections were counterstained with DAPI (4',6-diamidino-2-phenylindole) at 1 μg/ml for 5 min at room temperature (in the dark). The slides were finally washed again with 1x PBS twice, 5 min each, and mounted with Mowiol mounting medium. The slides were dried overnight in the dark and visualized under the microscope.

RNA isolation and qRT-PCR

RNA isolation was performed either by using an RNeasy lipid tissue kit as specified by the manufacturer or the 'crude' method. For the crude method, transfer the freshly harvested zebrafish brain(s) into a new 1.5-ml microcentrifuge tube (MCT). Here, we pooled five brains to prepare a sample. This protocol gives an acceptable yield (concentration) with a single brain also. The tissues were snap-frozen by dipping the MCT in liquid nitrogen and were then stored at −80°C. If liquid nitrogen is not available, proceed with the protocol immediately. The brains were homogenized in 200 μl commercially available TriZOL reagent initially, another 800 μl TriZOL was then added to the MCT and incubated at room temperature for 5–10 min. 200 μl chloroform was added to this followed by shaking vigorously by invert-mixing for 15–30 sec. Note: the suspension should not be vortexed. The mixture was then incubated for 5 min at room temperature with intermittent shaking followed by centrifugation at 12,000 g for 15 min at 4°C. The upper aqueous phase (around 400–450 μl) containing RNA was carefully transferred into a fresh 1.5 ml MCT. Note: the phases should not be disturbed in the process to ensure purity. Repeat this step if the phases get disturbed. An equal volume of ice-cold isopropanol was added to the MCT and invert-mixed for 15–30 sec followed by either incubating in ice for 5–10 min or at −80°C for 4–5 hrs. Both conditions give a good RNA yield for zebrafish brain(s) qualitatively and quantitatively. The mixture was then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was discarded, and the RNA pellet was washed twice by adding 200 μl 80% ethanol (v/v) and centrifuging at 12000 g for 5 min. The supernatant was discarded. Note: 80% (v/v) ethanol should be prepared in nuclease-free water. The RNA pellet should be air-dried after discarding the supernatant for 20–30 min. The RNA pellet was resuspended in nuclease-free water (25–30 μl for five brains pooled and 12 μl for 1 brain). The RNA was quantified using a Nanodrop UV Spectrophotometer and run on a 1% (w/v) agarose gel in 1xTBE (Tris-borate-EDTA). A good-quality RNA should have a 260/280 absorbance ratio of 2.0 and a 260/230 absorbance ratio between 1.5 and 2.0. On the agarose gel, two bands (28 s and 18 s) should be visible.

cDNA synthesis

Equal amount of RNA (1 μg) was taken from each sample to prepare cDNA using a cDNA synthesis kit. Make the RNA template mix as follows (Table 2):

The above mix was incubated at 65°C for 5 min and immediately incubated in ice for 5 min.

Add the following (see Table 3) to the RTM (RNA template mix):

The reaction on thermocycler was set as follows:

30°C—10 min
42°C—60 min
70°C—15 min

Nanodrop readings were taken, and the cDNA was kept at −20°C for long-term storage.

Table 2. RNA template mix for cDNA synthesis.

RNA	Volume for 1 μg
Random oligomers	1 μl
Oligo dT primers	1 μl
dNTP (deoxynucleotide triphosphate) mix	1 μl
Nuclease-free water	To make up the volume 11 μl

Table 3. Reaction components for cDNA synthesis.

RNA template mix	11 μl
Nuclease-free water	3.5 μl
Buffer (5x)	4 μl
Reverse transcriptase	1 μl
RNase inhibitor	0.5 μl
Total	20 μl

Quantitative real-time PCR (qRT-PCR)

Nanodrop readings of the cDNA synthesized are taken, which is then diluted to 100 ng/μl in nuclease-free water. This cDNA dilution is used in the qRT-PCR reaction. Five micromolar primer mix (Forward+Reverse) was prepared in nuclease-free water. Primer sequences used in the study are given in Supplementary Table S1. The master mix was prepared for each sample and primer combination in 2.5x reaction to account for pipetting errors. Master-mix preparation for 1x reaction is as follows (Table 4):

The master-mix was gently mixed by pipetting, and 15 μl master-mix from above was pipetted in each well. The plate was set in technical duplicates. 'No template control' for each primer pair was added as an additional control. The plate was finally sealed with plate sealer and covered with aluminium foil. The plate was spun at 1000 rpm for 2–3 min.

The reaction on the machine was set up as follows:

95°C – 20 sec
95°C – 15 sec
60°C – 30 sec
} 40 cycles

Melt curve analysis: 65–95°C (0.5°C increments at 2–5 sec/step). This step depends on the machine being used.

The data were analysed using delta-delta ct method [15], and the PCR products were run on a 0.8% (w/v) agarose gel in 1x TBE to check if correct products are obtained. The melt peak was checked to ensure that the primers are functioning as expected.

Results and discussion

Histological assessment of TBI-induced regeneration

The TBI model in zebrafish involves injuring one telencephalic hemisphere with a 30-g needle to induce regeneration, and the other uninjured telencephalic hemisphere acts as contralateral control (Fig. 1). To assess the histological changes that occur in the zebrafish brain during regeneration, classical haematoxylin and eosin (H&E) staining was used wherein haematoxylin

stains the nucleus and eosin stains the cytoplasm. Upon injury-induced regeneration, an accumulation of cells was observed in the injured telencephalic hemisphere at 7 dpl (days post-lesion). As the brain regenerates, we observed that the injury reduces in severity, as observed at 14 dpl, and largely diminishes at 30 dpl (Fig. 3). These results show overall morphology of the telencephalon upon injury and the process of restorative neurogenesis as the number of cells increases accumulating at the injury site in the regenerating telencephalic hemisphere. This model highlights the observed patterns of cell accumulation and injury resolution, which highlight the regenerative capacity of zebrafish.

Immunohistochemical analysis of TBI-induced regeneration

Upon regeneration, various cellular and molecular processes occur, which can be investigated using immunohistochemistry techniques. Key processes such as proliferation, gliogenesis, and neurogenesis during zebrafish brain regeneration can be studied using specific markers (antibodies). Following injury, intermediate progenitors proliferate and accumulate at the injury site to regenerate the lost tissue. An increase in the number of BrdU (bromodeoxyuridine)- and PCNA (proliferating cell nuclear antigen)-expressing cells was observed in the injured telencephalic hemisphere, indicating an enhanced proliferative response to generate new cells to compensate for the cell loss and/or damage due to injury (Fig. 4).

Furthermore, an increase in GFAP (glial fibrillary acidic protein), a marker for radial glial cells, was observed at both 7 dpl and 14 dpl (Fig. 5). Type II GFAP-expressing cells are considered to be actively proliferating cells which give rise to intermediate progenitors [8]. By 14 dpl, GFAP expression appears to be widespread throughout the parenchyma, reflecting the extensive glial response involved in the regenerative process. An understanding of the temporal and spatial patterns of the proliferation-specific and cell-specific markers during the regeneration process can greatly enhance our comprehension of the underlying mechanisms driving tissue repair and regeneration. This technique can be used as a vital tool for progressing regeneration-related research using zebrafish as a model organism.

Table 4. Reaction components for qRT-PCR.

SYBR	7.5 µl
Primer mix	0.3 µl
cDNA (100 ng/µL)	1 µl
Nuclease-free water	6.2 µl
Total	15 µl

Gene expression analysis upon TBI-induced regeneration

The TBI triggers a range of gene expression changes essential for mounting a functional regeneration response. To study these specific changes at the transcriptional level, quantitative real-time PCR (qPCR) was used. Ensuring the quality of RNA is critical for reliable and reproducible results. Ideal RNA quality is indicated by a 260/280 absorbance ratio of approximately 2.0, a 260/230 absorbance ratio between 1.5 and 2.0, and distinct 28S and 18S rRNA bands on an agarose gel. RNA isolation using both the kit procedures, and the crude method yielded high-quality RNA, as confirmed by 1% (w/v) agarose gel electrophoresis in 1x TBE (Fig. 6 A and B).

To investigate gene expression changes during zebrafish brain regeneration, markers such as *sox2* (stem cell marker), *olig2* (oligodendroglia marker) and *gfap* (glial cell marker) were used along with *rpl13a* which serves as a housekeeping gene. Upon comparing 7 dpl regenerating brains with control non-regenerating brain, an increase in the gene expression of *sox2, olig2,* and *gfap* was observed (Fig. 6 D). Further, to ensure the reliability of the results, the PCR products were run on the gel and expected product sizes were observed (Fig. 6 C).

Taken together, our results indicate that the protocols above offer a pipeline to investigate the process of zebrafish brain regeneration by employing histology, immunohistochemistry, and gene expression analysis. The protocols have been standardized for zebrafish brain but can also be repurposed for other tissues depending on the size and composition of the tissue. In our method, only the brain is processed for paraffin block preparation which minimizes additional steps that are needed when processing the brain with the skull. Further, it describes an efficient and easy method to prepare blocks such that coronal sections are obtained upon sectioning which is useful in telencephalic injury-induced regeneration model wherein the adjacent telencephalic hemisphere acts as contralateral control. Thus, our work contributes towards a cost-effective and reliable method to investigate brain regeneration process in zebrafish which is easily reproducible.

General notes

Paraffin block preparation and sectioning

- Four percent (w/v) paraformaldehyde once thawed from −20°C should be stored at 4°C and used within 7 days.

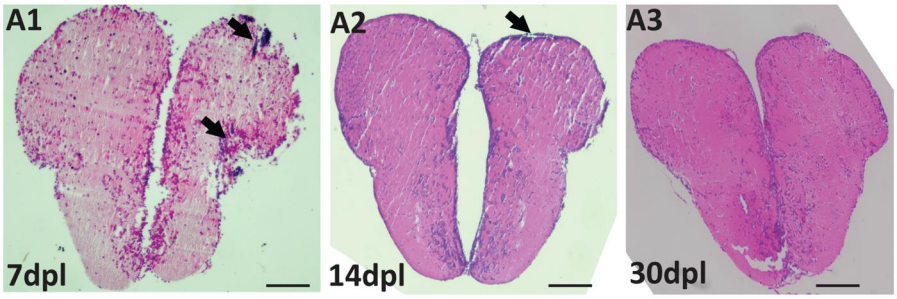


Figure 3. Histological assessment of the injury using HE staining at 7 dpl (A1), 14 dpl (A2) and 30 dpl (A3) (black arrow indicates the region of injury and accumulation of cells). Scale bar 100 µm.

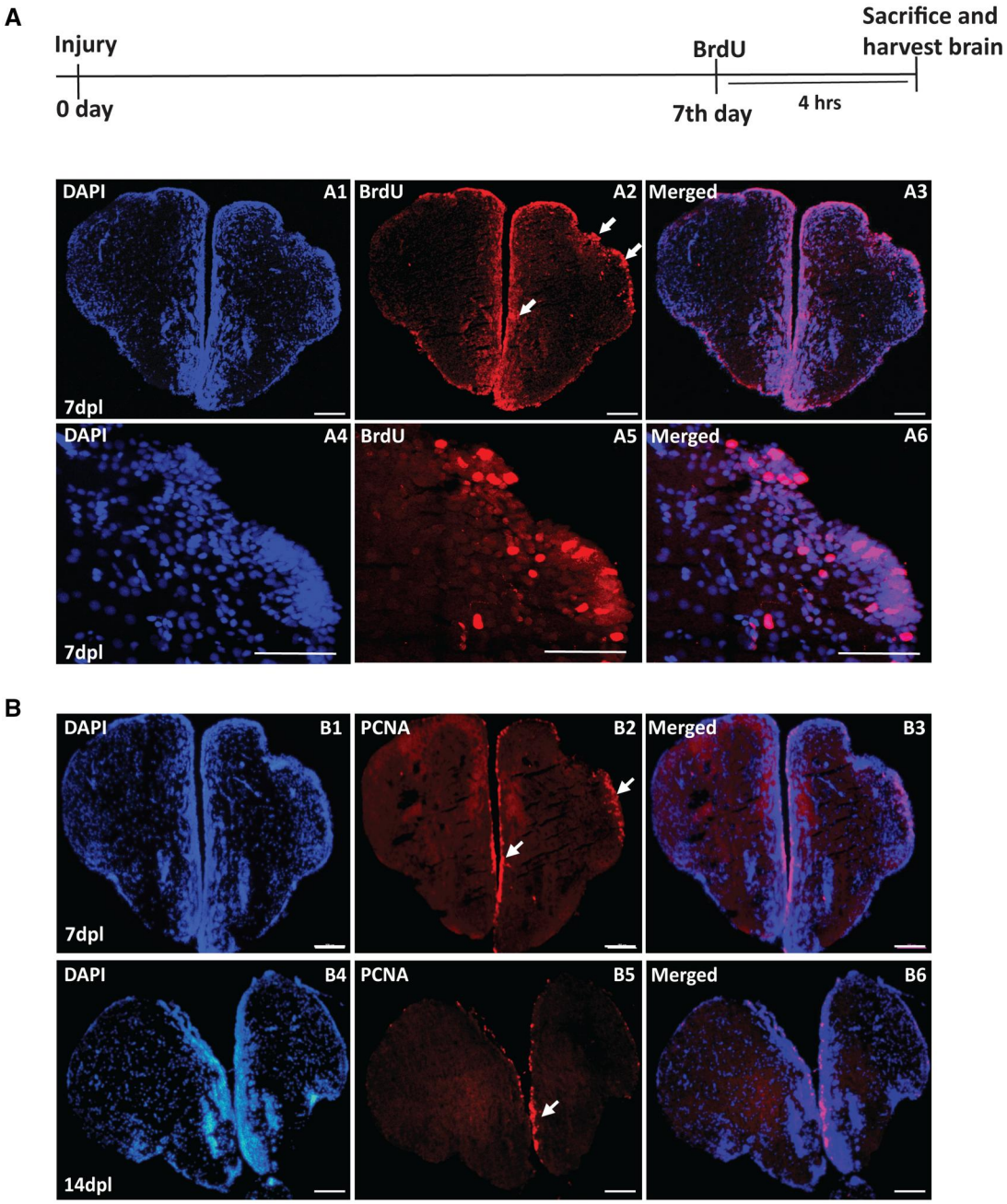


Figure 4. Immunostaining of regenerating brains showing increased proliferative response upon injury-induced regeneration. (A) Immunostaining of 7 dpl regenerating brain with anti-BrdU (A2) shows increased BrdU-positive cells (white arrows) in the injured/regenerating telencephalic hemisphere. Counterstaining is carried out with DAPI, and A3 shows merged image. (B) Immunostaining of 7 dpl and 14 dpl regenerating brain with anti-PCNA (B2 and B5) shows increased PCNA-positive cells (white arrows) in the injured/regenerating telencephalic hemisphere. Counterstaining is carried out with DAPI. B3 and B6 show the merged images. Scale bar 100 μm.

- Make sure the microtome blade is not too old/used. Using a sharp blade is essential for proper sections.

Histological stainings

- Make sure to use tap water wherever mentioned in the protocol.
- To improve eosin staining, add a few drops of acetic acid in the eosin solution.
- While staining with toluidine blue O, ensure the solution is on the section for no more than 45 sec for best results.

Immunostaining

- Prepare the sodium citrate solution fresh on the day of staining.
- 5 mM BrdU solution can be used 2–3 times, provided it is stored at −20°C.

RNA isolation and qRT-PCR

- Make sure the real-time primers are designed using NCBI BLAST, considering transcript levels at various zebrafish embryonic stages.

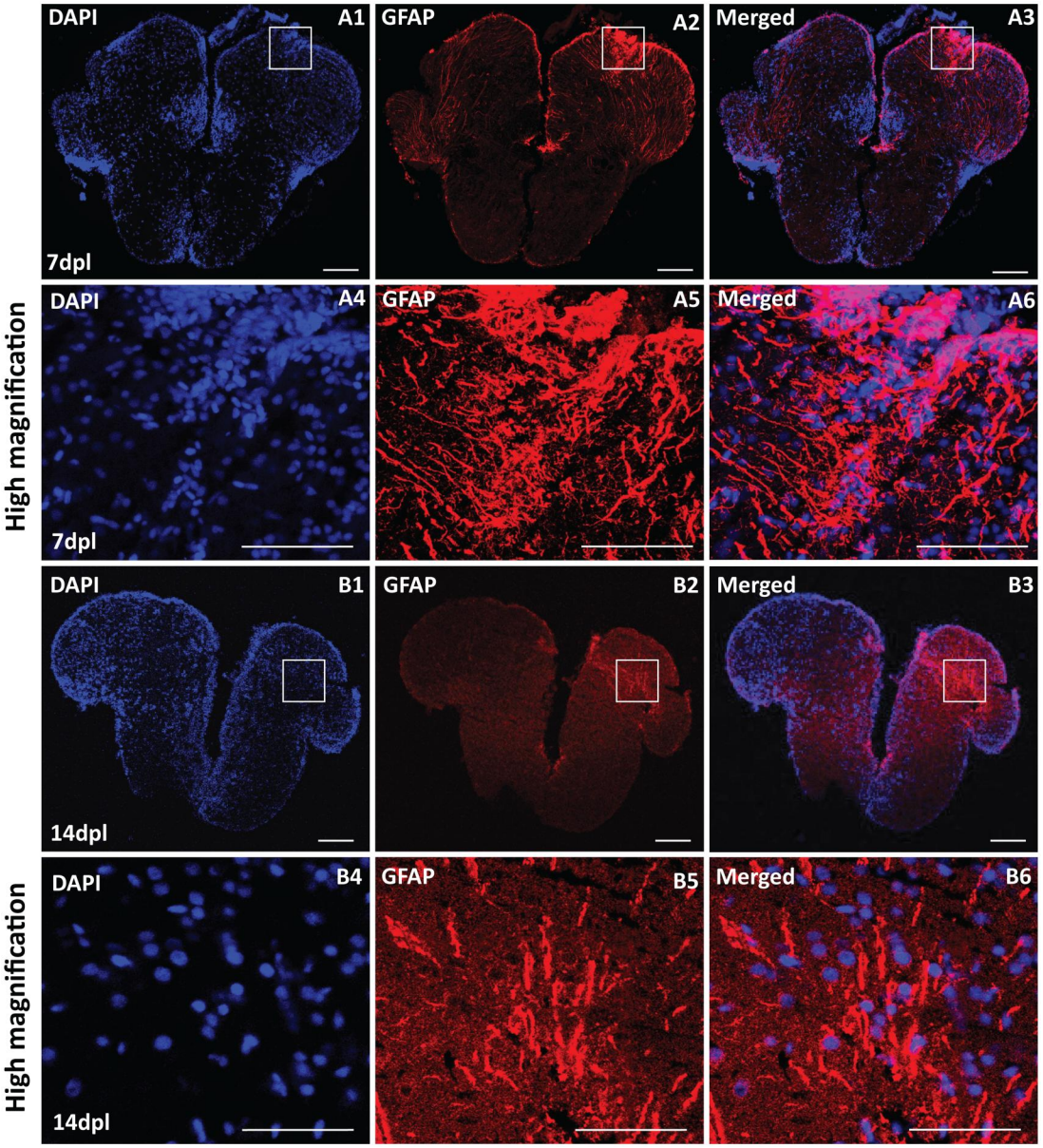


Figure 5. Immunostaining of regenerating brains showing increased glial response upon injury-induced regeneration. A2 and B2 show immunostaining of 7 dpl and 14 dpl regenerating brains with anti-GFAP (white square), suggesting increased GFAP-positive cells. Counterstaining is carried out with DAPI. A3 and B3 show merged images. The area highlighted in white squares are shown in the panel as high magnification images (A4–A6 and B4–B6). Scale bar 100 μm (A1–A3 and B1–B3), Scale bar 50 μm (A4–A6 and B4–B6).

- Synthesize full-length cDNA using the Takara kit. Avoid kits that only synthesize short transcripts.
- Thorough tissue lysis ensures high quantity of total RNA extraction from the brain samples.
- Each real-time primer set should be checked for a single amplification peak in the real-time reaction, and the melt curve should be run and verified for this each time. Post-real-time qPCR, samples can also be loaded into agarose gels to verify the presence of a single PCR product band of the required product size.

Data analysis

Immunostaining images were enhanced using Fiji, ensuring the dimmest cells were visible without saturating the brightest cells. The processing was applied evenly across the image. For qRT-PCR, the delta-delta ct method [15] was used to analyse the data, wherein rpl13a was used as a housekeeping gene. Standard error was used for error bars, and Student's t-test was used for statistical analysis using two-tailed distribution and two samples of equal variance.

Validation of protocol

Each experiment was performed with at least three biological replicates. For stainings, the uninjured hemisphere acts as a contralateral control. For qRT-PCR, three biological replicates of control and 7 dpl regenerating brains were used wherein each sample was prepared by pooling five brains. Student's t-test was

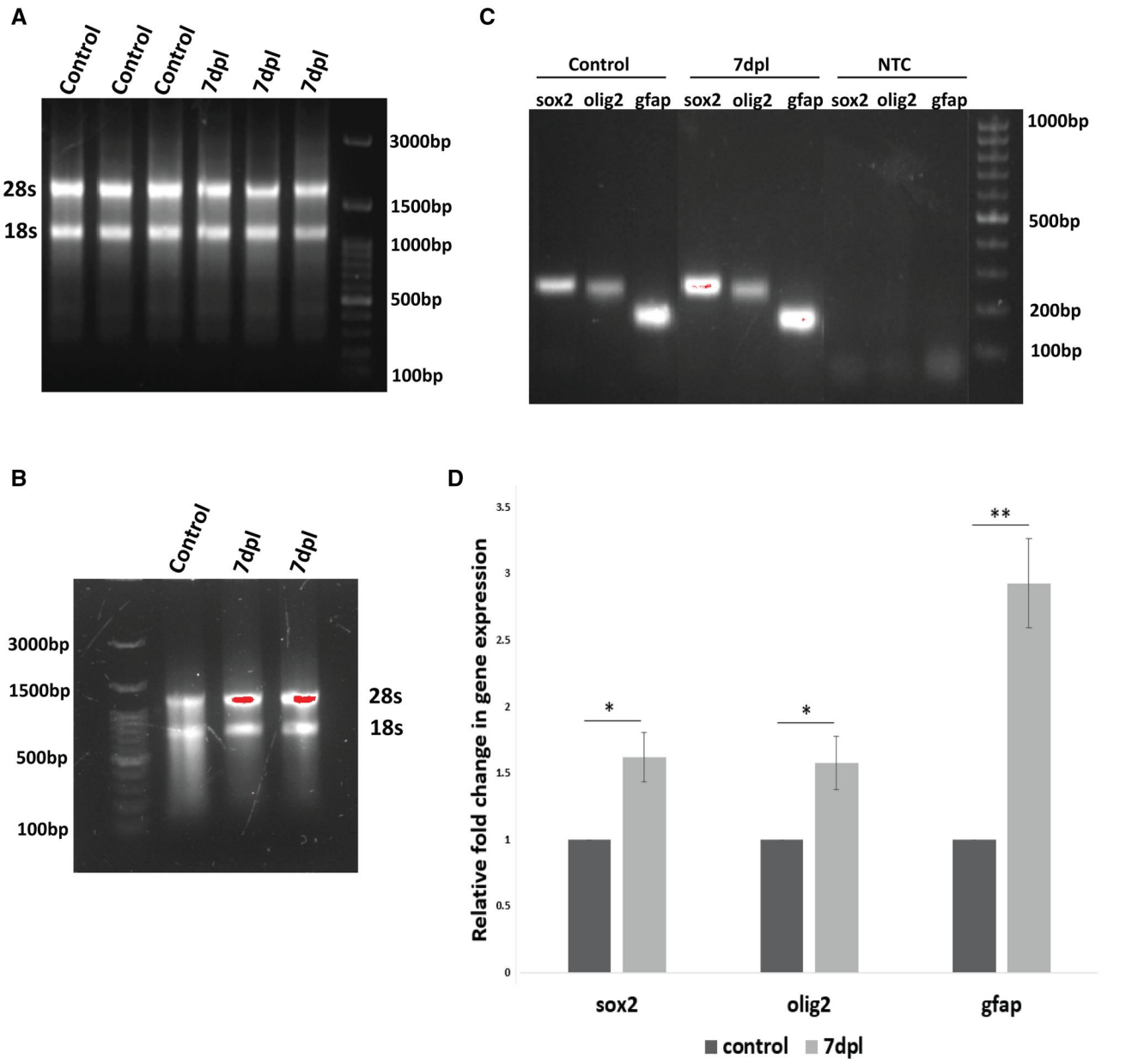


Figure 6. RNA isolation and qRT-PCR of regeneration-specific markers. (A) 1% (w/v) agarose gel in 1xTBE shows two RNA-specific bands (28 s and 18 s), and control and 7 dpl brain samples are shown in the gel followed by 1 kb ladder. (B) 0.8% (w/v) agarose gel in 1xTBE gel shows primer-specific PCR products upon RT-PCR (Sox2-240bp, *olig2*-224bp, and *gfap*-158bp). The control and 7 dpl samples show the correct product size for their respective primers. As expected, NTC (no template control does not give a product). Note: the gel is cut to show only relevant lanes. (C) Analysis of qRT-PCR using delta-delta ct method (*rpl13a* was used as housekeeping). p-value < 0.05 for *sox2* and *olig2*, p-value <0.01 for *gfap*.

used for statistical analysis for significance using two-tailed distribution and two samples of equal variance.

Acknowledgements

P.P.S. acknowledges IIT Delhi institute fellowship. S.M. acknowledges a Start-Up Research Grant from the Science and Engineering Board (SRG/2021/000341), a Ramalingaswami re-entry fellowship from the Department of Biotechnology (BT/RLF/Re-entry/70/2017), and IFCPAR/CEFIPRA (Indo-French Centre for Promotion of Advanced Research/Centre Franco-Indien pour la Promotion de la Recherche Avancée, grant no. 6503-J).

Author contributions

Priyanka Prakash Srivastava (Data curation [lead], Methodology [equal], Validation [lead], Writing—original draft [lead], Writing—review & editing [equal]), Sidharth Bhasin (Data curation [supporting], Methodology [equal], Writing—review & editing [equal]), Sunita Sathy Shankaran (Methodology [equal], Writing—review & editing [equal]), Catherine Roger (Methodology [equal], Writing—review & editing [equal]), Rajesh Ramachandran (Methodology [equal], Supervision [equal], Writing—review & editing [equal]), and Shilpi Minocha (Methodology [equal], Resources [lead], Supervision [lead], Writing—review & editing [equal])

Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

Conflict of interest statement. The authors declare that they have no conflict of interest.

Data availability

All the data generated during the study are included in the manuscript and the supplementary file. The list of primers, chemicals, and reagents used in this study along with the recipe of the buffers/solutions used in this study is also included in the supplementary file.

Ethical considerations

All animal experiments were carried out in accordance with the recommendations and protocols of the Institute Ethics Committee. The procedures were performed under anaesthesia. All efforts were made to minimize suffering.

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