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Whole Mount Immunostaining on Mouse Sciatic Nerves to Visualize Events of Peripheral Nerve Regeneration

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Whole mount immunostaining on mouse sciatic nerves to visualize events of peripheral nerve regeneration.

Xin-peng Dun and David B. Parkinson.

Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth, Devon, UK.

Address for correspondence:

Dr Xin-peng Dun, Plymouth University Peninsula Schools of Medicine and Dentistry, John Bull building, Derriford, Plymouth, Devon, PL6 8BU, UK.

E-mail: Xin-peng.Dun@plymouth.ac.uk

Running Head: Whole mount immunostaining of nerves to visualise repair.

i. **Abstract**

Injury to the peripheral nervous system triggers a series of well-defined events within both neurons and the Schwann cells to allow efficient axonal regeneration, remyelination and functional repair. The study of these events has previously been done using sections of nerve material to analyse axonal regrowth, cell migration and immune cell infiltration following injury. This approach, however, has the obvious disadvantage that it is not possible to follow, for instance, the path of regenerating axons in three dimensions within the nerve trunk or the nerve bridge. In order to provide a fuller picture of such events, we have developed a whole mount staining procedure to visualise blood vessel regeneration, Schwann cell migration, axonal regrowth and remyelination in models of nerve injury.

ii. **Key Words**

Whole mount immunostaining, peripheral nerve injury, axon regeneration, Schwann cell migration, blood vessel regeneration.

1. Introduction.

Whole mount *in situ* hybridization analysis or antibody labelling has been used in a number of developmental systems to study gene and protein expression in a variety of model organisms [1]. Immunolabelling of whole embryos or tissue followed by a simple tissue-clearing protocol and confocal microscopy allows cell migration, protein expression and tissue morphology to be visualised in three dimensions.

The events of Wallerian degeneration, axonal regeneration and functional repair following injury require interactions between many cell types, such as the neurons of the peripheral nervous system (PNS), Schwann cells, immune cells, endothelial cells and nerve fibroblasts [2-6]. These interactions lead to a directional migration of Schwann cells and regrowth of axons towards the distal part of the nerve, but it is often impossible to follow the paths of individual or groups of axons as they may well leave the plane of a thin cryostat or paraffin tissue section. To solve this problem, we have developed a modified whole mount staining protocol for sciatic nerves to analyse peripheral nerve repair following both nerve transection and crush models of injury [7]. This protocol has been used for our own research as well as successfully by other researchers in the field of peripheral nerve development and repair [8, 9]. In contrast to the 'two-dimensional' staining of thin cryostat or paraffin sections of peripheral nerves, the method is ideal for visualizing progression of blood vessel regeneration, Schwann cell migration, axonal regrowth and remyelination during the process of PNS regeneration following injury.

Figure 1 in this chapter shows a stained and mounted mouse sciatic nerve 5 days after transection injury. The proximal and distal stumps of the nerve together with the nerve bridge were labelled. Figure 2 shows the use of the whole mount immunostaining to visualize

blood vessels (marked with the endothelial cell marker CD31) and axon regeneration (marked with neurofilament, NF) 5 days after sciatic nerve transection injury. Figure 3 shows axonal regeneration (NF) and Schwann cell (S100β) staining in the nerve bridge 7 days after transection injury. Finally, Figure 4 shows the whole mount immunostaining using a transgenic mouse line, with a Schwann cell-specific expression of green fluorescent protein (PLP-EGFP mice) [10], together with a neurofilament stain to visualize both axonal regeneration and Schwann cell migration.

2. Materials

2.1 Mouse sciatic nerve injury, nerve dissection and fixation

1. Isoflurane anaesthetic set up for small animal surgery.
2. Thermostatically heated surgical pad.
3. Small animal hair clipper.
4. Forceps (#5 size, eg. Fine Science Tools (FST), Cat. No. 11251-10; 2 pairs).
5. Scissors for skin cut and separation of muscles (12cm eg.14002-12; 15003-08 | VannasTübingen Spring Scissors Straight).
6. 0.4mm tip angled, 9cm delicate forceps (e.g. FST, Cat no. 11063-07).
7. Needle holder for use with silk sutures (eg. World Precision Instruments, Cat no. 500223).
8. 8.0 size silk sutures.
9. 0.025% bupivacaine solution: prepare 1/10 dilution of 0.25% stock (e.g. Marcaine) in sterile PBS.
10. Autoclip surgical staple applier (e.g. FST, Cat no. 12020-09).
11. 70% alcohol in a spray bottle.

12. Corkboard.
13. Aluminium foil.
14. 7 ml screw top bijoux containers.
15. Dissecting microscope.
16. 4^{o}C fridge.
17. Phosphate buffered saline (PBS): 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, dissolve the reagents in 800 ml of water. Adjust the pH to 7.4 with HCl and then add water to 1 litre.
18. Fixing solution: 4% (w/v) paraformaldehyde (PFA) prepared in PBS, pH 7.4. Paraformaldehyde powder is dissolved in warmed (50^{o}C) PBS on a magnetic stirrer and add a few sodium hydroxide pearls or drops of 1M sodium hydroxide solution to help the powder dissolve. Allow the solution to cool down to room temperature and then re-adjust the pH if necessary to pH 7.4 with HCl. The solution can be stored at 4^{o}C for 2-3 months or aliquoted and frozen at -20^{o}C for long term storage.

2.2 Whole mount staining of mouse sciatic nerve tissue

1. PBS (see Section 2.1, Step 17)
2. Permeabilisation/blocking solution: 1% Triton X-100 detergent (v/v) and 10% (v/v) foetal bovine serum prepared in PBS. Make up and store at 4^{o}C.
3. Staining solution: 0.1% Triton X-100 detergent (v/v) and 10% (v/v) foetal bovine serum prepared in PBS. Make up and store at 4^{o}C.
4. Primary antibodies.
5. Appropriate fluorescently labelled species-specific secondary antibodies.
6. Clearing solutions: 25%, 50% and 75% glycerol (v/v) prepared in PBS.
7. Glass slides.
8. Vacuum grease.

9. CitiFluor anti-fade mounting medium.
10. 20mm x 40mm glass coverslips.
11. Nail varnish.
12. Epifluorescence and confocal microscope.

3. Methods

3.1 Mouse sciatic nerve transection or crush injury

Sciatic nerve crush or cut procedures are performed on adult wild-type or transgenic mice, usually between 2 and 3 months of age (weight greater than 20g).

1. Prepare clean cages and labels for mice undergoing surgery.
2. Prepare a sterile surgical area with drapes, the anaesthesia machine, sterilised surgical tools, a diluted bupivacaine solution (1/10 dilution of 0.25% stock in sterile PBS to make 0.025% preparation for use) and heating pad in the designated surgery room.
3. Anaesthetise the animals with isoflurane in the anaesthesia box.
4. Transfer the mouse to the mask and remove the hair around the incision area with a hair clipper.
5. Use sticky tape to gently secure the rear legs on heating pad and test the depth of anaesthesia by checking the toe reflex to ensure the animal is fully anaesthetised.
6. Identify the position for incision on the mid-thigh of the animal and perform a skin cut (approximately 1cm in length) using scissors.
7. Carefully separate the gluteal and the hamstring muscles with scissors and forceps to expose the sciatic nerve.
8. Perform a sciatic nerve crush or cut 3mm proximal to the trifurcation site of sciatic nerve (*see* **Note** 1). For nerve crush experiments, use a pair of delicate forceps (Fine Science Instruments; 0.4mm tip angled, Cat no. 11063-07) and crush the sciatic nerve once for 30

seconds, and again for 30 seconds at the same site but orthogonal to the initial crush. For a nerve cut, the nerve is transected using fine scissors at the same point as for crush.

9. Close the gluteal and the hamstring muscles using an 8.0 suture and topically apply one drop of 0.025% bupivacaine solution above the muscle for local analgesia.
10. Close the skin with a surgical clip using an Autoclip applier.
11. Weigh the mouse and put the mouse in the heated recovery box.
12. Wait until the mouse fully wakes up and then return back to the cage.
13. Repeat step 3 to 12 for the next mouse undergoing surgery.
14. Check the mice that have undergone surgery each day. The weight/condition of the animals post-surgery should be checked and recorded daily until they are euthanased for analysis.

3.2. Dissection and fixation of mouse sciatic nerve tissue.

1. Clean the dissecting area with 70% (v/v) alcohol/water.
2. Cover a corkboard with tin foil and then wash with 70% alcohol.
3. Fill the 7 ml screw top bijoux containers with 5ml 4% PFA.
4. Kill mice by using the appropriate approved procedure such as using carbon dioxide inhalation and/or cervical dislocation.
5. Pin the mouse carcass out onto a cork board and spray the mouse body with 70% alcohol to keep the fur wet and to not interfere with the dissection.
6. Use forceps and scissors and remove the skin from lower part of the mouse's body.
7. Dissect out the sciatic nerves (*see* **Note** 2 and 3) and fix them in 4% PFA for a minimum of 5 hours (h) up to 24h at 4^{o}C.
8. Clean the dissecting area and dissecting tools.

3.2 Permeabilisation, blocking and staining of samples

1. Following fixation, nerve samples are washed three times in PBS, each wash lasting ten minutes, at room temperature in a 7 ml screwtop bijoux container (*see* **Note** 4 and 5 for tissue preparation and multiple nerve staining). Nerve tissue samples are then permeabilised and blocked with permeabilisation/blocking solution (see 2.2 Solutions) overnight at 4^{o}C to prevent non-specific binding of primary antibody.
2. Desired primary antibodies (*see* **Note** 6) are then diluted into staining solution and incubated with the nerve sample for 72 hours at 4^{o}C to allow primary antibodies to fully penetrate into the nerves.
3. Following incubation with primary antibody, nerve samples are then washed three times in PBS at room temperature, each wash last for 15 minutes. After these three 15 minute washes, the nerves are then washed using a further six changes of PBS, one hour each wash, to ensure complete removal of unbound primary antibody.
4. Nerve samples are then incubated for 48h at 4^{o}C with fluorescently labelled species-specific secondary antibodies diluted into staining solution Hoechst dye to stain nuclear DNA can also be added to the secondary antibody solution at this point (*see* **Note** 6 for concentration of secondary antibody and Hoechst dye and **Note** 7 for the monitoring of secondary antibody binding).
5. Excess secondary antibody is then removed by washing in PBS for 6 h at room temperature, changing the PBS each hour.
6. Nerve samples are then cleared for imaging with increasing concentrations of glycerol at 25%, 50% and 75% (v/v) glycerol in PBS. The stained nerve is placed in 25%, 50% and 75% glycerol solutions sequentially, incubated 24 hours at 4^{o}C for each increasing glycerol concentration. The cleared nerve tissue is then ready to be mounted onto a glass slide for imaging (*see* **Note** 8 and 9).

3.3 Mounting and imaging of whole mount stained nerve

Following completion of the whole mount immunolabelling, the nerve tissue is now ready to be mounted onto a glass slide for imaging.

1. Place the stained nerve tissue in the centre of the glass slide and gently pull both ends of nerve tissue with forceps to keep the nerve tissue straight (*see* **Note** 10).
2. Apply vacuum grease on the glass slide around the four corners where the glass coverslip will be placed（we would usually use 22mm×40mm glass coverslips）.
3. Place a coverslip on the top of the vacuum grease and apply a gentle pressure to allow the coverslip to just contact the nerve on the glass slide.
4. Apply CitiFluor anti-fade mounting medium on one side of the coverslip to allow penetration through the whole area covered under the glass coverslip.
5. Seal the coverslip around using clear nail varnish and allow the varnish to fully dry (*see* **Note** 11 for storage).
6. Stained and cleared nerves are imaged using a confocal microscope and several Z-series stacks are captured covering the entire field of interest. Individual series may then be flattened into a single image for each location and then combined using image analysis software such as Adobe Photoshop (Figures 2, 3 and 4). 3D imaging and reconstructions may also be generated using appropriate 3D software such as Fiji and Imaris software.

4. Notes

1. In order to visualize blood vessel regeneration, Schwann cell migration and axonal regeneration in the nerve gap, no re-anastamosis of the severed nerve, either by suture or by fibrin glue, is performed in the nerve transection procedures. Without re-anastamosis,

a nerve bridge will be formed between the proximal and distal stumps of the nerve 4 days following transection (Figure 1) [11]. The nerve bridge length may vary between 1.5 and 2.5 mm in the mouse [7].

2. For analysis of repair following nerve crush injury, the entire sciatic nerve proximal and distal to the injury, including tibial, peroneal and sural branches are carefully dissected as distally as possible and the intact nerve tissue. To ensure good antibody penetration into the nerve post-fixation, using fine forceps, the epineurium is carefully removed from the entire length of nerve tissue.
3. During dissection, great care must be taken to not damage the delicate bridge tissue formed between the proximal and distal nerve stumps following nerve transection. In order to preserve the nerve bridge tissue, the sciatic nerves (3-5mm proximal and distal to the injury site) together with the muscles underlying the nerve bridge are dissected out for fixation. Muscle tissue still attached to the nerve will be removed after the paraformaldehyde fixation.
4. The epineurium in the distal nerve stump prevents antibody penetration and it needs to be removed in order to visualize axonal regeneration and remyelination in the distal nerve stump. However, the blood vessel regeneration, Schwann cell migration and axonal regeneration can be visualized in the nerve bridge without removing the epineurium.
5. For samples of transected nerves, several nerve preparations can be stained in the same 7ml bijoux container. For crush injury, we normally dissect out a segment of nerve longer than 2cm and it is better to process the nerve crush samples separately, otherwise the nerves often tangle with each other.
6. For whole mount staining, both primary and secondary antibodies are normally used at twice the concentration as used for immunohistochemistry on cryostat sections or cell labelling on coverslips. This increased concentration allows full penetration of the

antibodies into the nerve tissue and bridge. Hoechst dye for staining of nuclear DNA is used at the same concentration as for standard immunohistochemistry or cells on coverslips.

7. The binding of secondary antibody can be checked under the epifluorescence microscope daily.
8. Following whole mount staining, surrounding tissue around the nerve bridge can be further removed under the epifluorescence microscope after the 75% glycerol clearing step. This may improve the quality of imaging for the sample.
9. For samples that have undergone crush injury, the crush site is recognizable on the slide by eye after the 75% glycerol clearing step; the crush site is always more transparent than the rest of the tissue. We use this method to measure from the crush site to the furthest growing axon and study the speed of axon regeneration in the mouse. The longest time point that we have used for this method is 7 days following crush injury as leading axons are still visible in the tibial nerve in C57BL/6 mice. At later timepoints, axons have regenerated fully from the crush site through the entire distal nerve and the relative axonal regeneration rates between different mouse lines cannot be measured.
10. The diameter of mouse sciatic nerve varies between mouse lines and even between male and female mice. For two month old control C57BL/6 mice, the sciatic nerve is normally between 0.6-0.8mm in diameter.
11. Mounted slides of whole mount stained nerves can be stored at 4^{o}C. For staining with antibodies such as neurofilament, the fluorescence signal is visible even up to 1 year after staining. However, background signal and auto-fluorescence do appear to increase over time with storage.

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Figure Legends:

Figure 1: Phase contrast image of a stained and mounted mouse sciatic nerve. The adult mouse (C57BL/6) nerve sample was dissected at 5 days post-transection injury. The presence of the newly-formed nerve bridge between the proximal and distal stumps can easily be visualised at this time point.

Figure 2: Visualization of blood vessel and axon regeneration in the mouse sciatic nerve bridge at 5 days following a transection injury. Whole mount immunostaining of mouse sciatic nerve at 5 days post-transection injury, new blood vessel formation within the nerve bridge was labelled with endothelial cell marker CD31, existing blood vessels within the proximal and distal nerve stump also have been labelled with CD31. At this time point, regenerating axons from the proximal stump could be visualized with neurofilament (NF) staining (white arrow) and axonal breakdown in the distal stump is complete.

Figure 3: Visualization of Schwann cell migration and axonal regeneration in the mouse sciatic nerve bridge 7 days after transection injury. (A) Labelling of regenerating axons using neurofilament (NF) shows axons crossing the bridge into the distal stump at this timepoint. Labelling with S100β shows Schwann cells migrating into the nerve bridge from both proximal and distal stumps. (B) Overlay of phase contrast image and fluorescence shows

the nerve structure. The positions of the nerve bridge, the proximal and distal nerve stumps are shown.

Figure 4: Visualization of Schwann cell migration and axonal regeneration in the sciatic nerve bridge of PLP-EGFP transgenic mice at 6 days following transection injury. PLP-EGFP mice express an enhanced green fluorescent protein (EGFP) under the control of the Schwann cell-specific proteolipid protein (PLP) promoter [10]. (A) Whole mount immunostaining with the neurofilament (NF) antibody shows regenerating axons from the proximal nerve stump localised in the front of migrating Schwann cells at 6 days after sciatic nerve transection injury. Migrating EGFP-positive Schwann cells from the distal nerve stump could also be clearly observed. Positions of the nerve bridge, the proximal and the distal nerve stumps are shown. (B) Higher magnification image shows regenerating axons from the proximal nerve stump are localised in the front of migrating Schwann cells at 6 days after sciatic nerve transection injury. (C and D) Higher magnification images show Schwann cell migration toward the nerve bridge from both the proximal (C) and the distal (D) nerve stumps.