Video Article

Investigating Mammalian Axon Regeneration: *In Vivo* Electroporation of Adult Mouse Dorsal Root Ganglion

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URL: https://www.jove.com/video/58171
DOI: doi:10.3791/58171

Keywords: Neuroscience, Issue 139, Electroporation, dorsal root ganglion, DRG, *in vivo*, genetic manipulation, sensory neuron, axon regeneration, peripheral nerve injury, non-viral transfection

Date Published: 9/1/2018

Citation: Li, Q., Qian, C., Zhou, F.Q. Investigating Mammalian Axon Regeneration: *In Vivo* Electroporation of Adult Mouse Dorsal Root Ganglion. *J. Vis. Exp.* (139), e58171, doi:10.3791/58171 (2018).

Abstract

Electroporation is an essential non-viral gene transfection approach to introduce DNA plasmids or small RNA molecules into cells. A sensory neuron in the dorsal root ganglion (DRGs) extends a single axon with two branches. One branch goes to the peripheral nerve (peripheral branch), and the other branch enters the spinal cord through the dorsal root (central branch). After the neural injury, the peripheral branch regenerates robustly whereas the central branch does not regenerate. Due to the high regenerative capacity, sensory axon regeneration has been widely used as a model system to study mammalian axon regeneration in both the peripheral nervous system (PNS) and the central nervous system (CNS). Here, we describe a previously established approach protocol to manipulate gene expression in mature sensory neurons *in vivo* via electroporation. Based on transfection with plasmids or small RNA oligos (siRNAs or microRNAs), the approach allows for both loss- and gain-of-function experiments to study the roles of genes-of-interests or microRNAs in regulation of axon regeneration *in vivo*. In addition, the manipulation of gene expression *in vivo* can be controlled both spatially and temporally within a relatively short time course. This model system provides a unique tool to investigate the molecular mechanisms by which mammalian axon regeneration is regulated *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/58171/

Introduction

Injuries in the nervous system caused by neural trauma or various neurodegenerative diseases usually result in defects in motor, sensory and cognitive functions. Recently, much effort has been devoted to regenerative potency re-establishment in adult neurons to restore the physiological functionsof injured neurons[1,2,3]. Sensory neurons in the DRG are a cluster of nerve cells that convey different sensory stimuli, such as pain, temperature, touch, or body posture, to the brain. Each of these neurons is pseudo-unipolar and contains a single axon that bifurcates with one branch extending toward the periphery and the other branch heading toward the spinal cord[4]. The adult sensory neurons in DRGs are among a few mature mammalian neurons known to regenerate their axons actively after injury. Hence, injuries of sensory axons have been extensively employed as a crucial model to study the mechanisms of axonal regeneration *in vivo*.

In vivo gene transfection techniques, which are usually less time-consuming to set up and more flexible than using transgenic animals, have been playing essential roles in studying the functions of genes and signaling pathways in the nervous system. The main techniques can be categorized into two approaches: instrument-based and virus-based[5]. Viral-based *in vivo* gene delivery in adult neurons can provide precise spatiotemporal manipulation of gene expression[6]. However, labor-intensive processes are involved in viral-based methods, such as the production and purification of viral particles containing the desired gene. In addition, many viral vectors could activate the immune system of the host, which may interfere with the data acquisition, data analysis and possibly mislead the interpretation of experimental results. Electroporation, a typical instrument-based transfection approach, uses an electrical pulse to increase the permeability of cell and nuclear membranes transiently, which favors the influx of gene vectors or small RNA oligos from space outside of the cells[7]. *In vitro* electroporation is widely recognized as a transient but highly efficient strategy for manipulating targeted gene expression in many cell types. Although *in vivo* electroporation only leads to transient gene expression with low transfecting efficiency compared to viral vectors, it has various advantages over viral approaches. For instance, it can be applied to almost all tissues and cells[7,8,9]. In addition, either plasmids encoding genes-of-interest or small RNA oligos (*e.g.,* siRNAs, microRNAs) against certain transcripts can be injected into the target tissue directly and then electrically pulsed, which make the procedure less labor- and time- consuming. Moreover, transfecting multiple plasmids and RNA oligos simultaneously with single electroporation is possible.

We have established an *in vivo* electroporation approach to manipulate gene expression in adult mouse sensory neurons and successfully applied and validated such approach in numerous pioneer studies[1,2,3,8,10]. Here, we present a detailed protocol to facilitate the usage of this approach for future studies of mammalian axon regeneration.

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Protocol

All animal experiments were performed in accordance with the animal protocol approved by the Johns Hopkins Institutional Animal Care and Use Committee.

1. Materials and Reagents

1. **Animals**
    1. Use six-week-old female CF1 mice weighing 30–35 g for the experiments.
    NOTE: The mice were group-housed (5 mice per cage) in individually ventilated sterilized cages with 1/4" corn cob bedding and 2" square nestlets for nesting. The cages were maintained in a controlled 12-h light-dark cycle and the room temperature was between 19.4 °C and 25 °C. The mice were fed with global rodent diet and automatic water supplying system.

2. **Instruments**
    1. Use the following surgical instruments to efficiently conduct the surgery: disposable syringes (27 G, 1.0 mL) for intraperitoneal injection, fur clipper, stereo dissection microscope, micro-surgery forceps, micro-surgery scissors, and small bone rongeurs and sterilized non-woven sponges.
    NOTE: All the instruments and materials are autoclaved before surgery or pre-sterilized by the producers. During surgery, the instruments are sprayed with 75% alcohol to maintain the sterilization.

3. **Anesthetic Solutions**
    1. Use anesthetic solution #1 to induce the anesthesia and use anesthesia solution #2 before DRG injection to maintain the anesthesia.
        1. To make anesthetic solution #1, dilute ketamine and xylazine in sterile saline at a final concentration of 10 mg/mL and 1.2 mg/mL.
        2. To make anesthetic solution #2, dilute avertin in sterile saline at a final concentration of 20 mg/mL.

4. **DNA Plasmid and RNA Oligos**
    1. Prepare the plasmids using the commercial kit following the manufacturer's protocols accordingly. Dilute the plasmid DNAs in sterile deionized water to the concentration of 2.0 μg/μL.
    2. Add Fast Green dye solution to reach a final concentration of 0.005–0.01% (v/v) for better visualization of the DRG outline during injection.
    3. Dissolve the siRNA or microRNA oligos in corresponding buffers provided by manufacturers to reach a final concentration at 50 μM.

5. **Micro-Injection Pipette**
    1. Pull the capillary glass using the micropipette puller and cut the tip of the pulled capillary glass pipette with microsurgery scissors to generate an opening with an approximate 50 μm outer diameter. Then sterilize the glass pipette under UV light on a clean bench for 20–30 min approximately.
    2. Mount the sterilized glass pipette on the pipette holder connected to an intracellular microinjection dispense system. Set the parameters of the microinjection system at 30 psi of pressure and 8 ms of duration.

6. **Electroporation System**
    1. Connect the electrodes to the square wave electroporation system and set the following parameters: 15 ms pulses at 35 V with 950 ms intervals for *in vivo* electroporation.

7. **Perfusion and Fixing Solution**
    1. Dissolve the paraformaldehyde (PFA) powder in 1x PBS at a concentration of 4% (w/v). Store the PFA solution at 4 °C.

2. Experimental Procedures

1. **Surgical Exposure of the L4 and L5 DRGs**
    1. Anesthetize the mouse using an intraperitoneal injection of the anesthetic solution #1 prepared previously (with ketamine 100 mg/kg body weight and xylazine 12 mg/kg body weight).
    2. Shave the surgical area with the fur clipper.
    NOTE: Since there will be another surgery for left sciatic nerve crush, the fur of the left posterior thigh can be shaved simultaneously.
    3. Place the mouse on the heated blanket (35.0 °C) and monitor the rectal temperature with a temperature probe.
    4. To test the anesthesia, pinch the toes and tail of the mouse and observe the behavioral responses.
    5. Tape the four limbs of the mouse on the corkboard.
    6. Wipe the surgical area with iodophor solution and then with 75% alcohol to remove the iodophor before cutting the skin.
    7. Apply ophthalmic ointment on eyes to prevent dryness while under anesthesia.
    8. Before incision, mark both sides of the iliac crests with a fine marker pen. Draw a line connecting two points of iliac crests to facilitate identifying the positions of L5 DRGs.
    9. Make a 3 cm incision along the midline of low back with micro-scissors. Moreover, detach the paraspinous muscles, such as the musculus multifidus and the musculus longissimus lumborum, from the L3 to S1 spinous processes, and expose the facet joints of L4-5 and L5-6.

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10. Use a micro-rongeur to remove the facet joints of L4-5 and L5-6. Moreover, remove the left neural arch of L4 and L5 to expose the dorsal side of the DRGs.

2. **DRG Injection**
    1. Inject anesthetic solution #2 (with avertin 200 mg/kg body weight) intraperitoneally to maintain the anesthesia before DRG injection.
    2. Load the DNA plasmids or RNA oligos (1 µL per DRG) into the glass capillary pipette.
    3. Insert the tip of the capillary glass pipette carefully into the DRG.
    4. Gradually inject 1.0 µL solution of DNA plasmids or RNA oligos into the DRG using the intracellular microinjection dispense systems (30 psi, 8 ms).
       NOTE: The duration of the injection should last no less than 5 minutes.

3. **Electroporation**
    1. Drop PBS on tips of the electrodes.
    2. Clean up the bleeding with sterilized square cotton gauze.
    3. Pinch the target DRG with the electrodes gently and apply 5 square electric pulses with the electroporation system.
    4. Close the muscle and skin layers respectively with 5-0 nylon sutures.
    5. Place the mouse on a heated blanket (35.0 °C) under close attention until it has completely regained sufficient consciousness to maintain sternal recumbency. Return the mouse to the home cage after it has fully recovered from the anesthesia.
       NOTE: It takes approximately 60 min for the mouse to completely recover from anesthesia on the 37 °C blanket. Dissolve one pill (200 mg) ibuprofen into 1,000 ml water (0.2 mg/ml) for daily feeding to relieve the post-surgical pain.

4. **Sciatic Nerve Crush**
    1. Two or three days (depending on the experimental design) after the DRG electroporation, anesthetize the mouse intraperitoneally with anesthetic solution #2 (with avertin 400 mg/kg body weight).
    2. Tape the four limbs of the mouse on the corkboard.
    3. Make a 1 cm incision 0.5 cm to the left side along the midline. Cut the muscles, such as gluteus maximus muscle and piriformis muscle, longitudinally. Expose the segment of the sciatic nerve between the greater sciatic foramen and the sciatic notch.
    4. Crush the nerve with microsurgery forceps for 12 s and mark the crush site with a 10-0 nylon epineural suture. Make a knot on the dural membrane to mark the crush site.
    5. Close the muscle and skin layers with 5-0 nylon suture.
    6. Place the mouse on a heated blanket (35.0 °C) with close attention until it has completely regained sufficient consciousness to maintain sternal recumbency. Return the mouse to the home cage after it has fully recovered from the anesthesia.
       NOTE: It takes approximately 60 min for the mouse to completely recover from anesthesia on the 37 °C blanket. Dissolve one pill (200 mg) ibuprofen into 1,000 ml water (0.2 mg/ml) for daily feeding to relieve the post-surgical pain.

5. **Mouse Perfusion, DRG and Sciatic Nerve Harvest**
    1. Two or three days after the sciatic nerve crush (depending on the experimental design), anesthetize the mouse intraperitoneally with anesthetic solution #2.
    2. Perfuse the mouse transcardially with PBS (pH 7.4) followed by ice-cold 4% paraformaldehyde (PFA) (pH 7.5) in PBS.
    3. After perfusion, separate the DRGs together with the nerve roots and sciatic nerve before the knee carefully with micro-scissors and micro-forceps under the dissection microscope. Place the sciatic nerve directly in 4% PFA overnight at 4 °C.

6. **Imaging and Measuring the Fluorescence-Labeled Sensory Axons**
    1. Strip off the attached tissue and membrane on the fixed sciatic nerve with micro-scissors and micro-forceps carefully under the dissection microscope. Then, exchange the 4% PFA with PBS and wash the nerve three times.
    2. Place the sciatic nerve on a slide and keep it straight. Add 80 µL of antifade solution around the nerve, then lay a coverslip on it. Flatten the whole mounted tissue with pressure.
    3. Place the flattened tissues on the inverted epifluorescent microscope equipped with an accessory for mosaic acquisition and image processing.
       NOTE: The excitation and emission lengths are 488 nm and 509 nm. It is also possible to take multiple overlapping images manually and stitch the images together using ImageJ with the Mosaic plug-in.
    4. When measuring the length of regenerated axons, trace all identifiable fluorescently-labeled axons in the sciatic nerve from the crush site (marked with the 10-0 epineural suture) to the distal axon ends.

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Representative Results

To quantify the cytotoxicity of the current protocol and to validate that transfection rate of *in vivo* DRG electroporation is high enough, we injected and electroporated fluorescently-tagged microRNA or siRNA into L4 and L5 DRGs. The detached DRGs were processed through cryo-sectioning and immunohistochemistry (**Figure 1A-B**). When estimating the cell survival rate after injection and electroporation, the intact DRGs from L4 and L5 were harvested and processed through cryo-sectioning and immunohistochemistry. The neuron densities, reflected with Tuj1 staining, did not show a significant difference when compared to the neuron densities of intact DRGs, which indicates that the electroporation did not induce neuronal cell death (**Figure 1C**). The transfection rate was calculated as the ratio between the number of tagged RNA oligo neurons and the number of Tuj1-positive neurons. The mean transfection rate of miRNA is 80.7 ± 4.3% and the siRNA 94.2 ± 0.3% (**Figure 1D**). When applying the current *in vivo* electroporation method to study axon regeneration, the sciatic nerve was flattened and imaged. Every regenerated axon with distinctive trajectory and recognizable distal axon end can be traced from the crush site indicated by the suture knot (**Figure 2**). Additionally, we electroporated the DRGs with EGFP plasmids and harvested the spinal cord from T7 to L2 along with dorsal roots of L4 and L5 after 7 days. We used a well-established tissue clearing protocol — uDISCO to clear the spinal cord[11]. The EGFP-labeled dorsal column axons in the spinal cord were imaged with a confocal microscope and then identified in both the longitudinal and the sagittal projecting images (**Figure 3**). Alternatively, the confocal images can be processed with microscopy visualization software to reconstruct a 3D image (**Movie 1**).

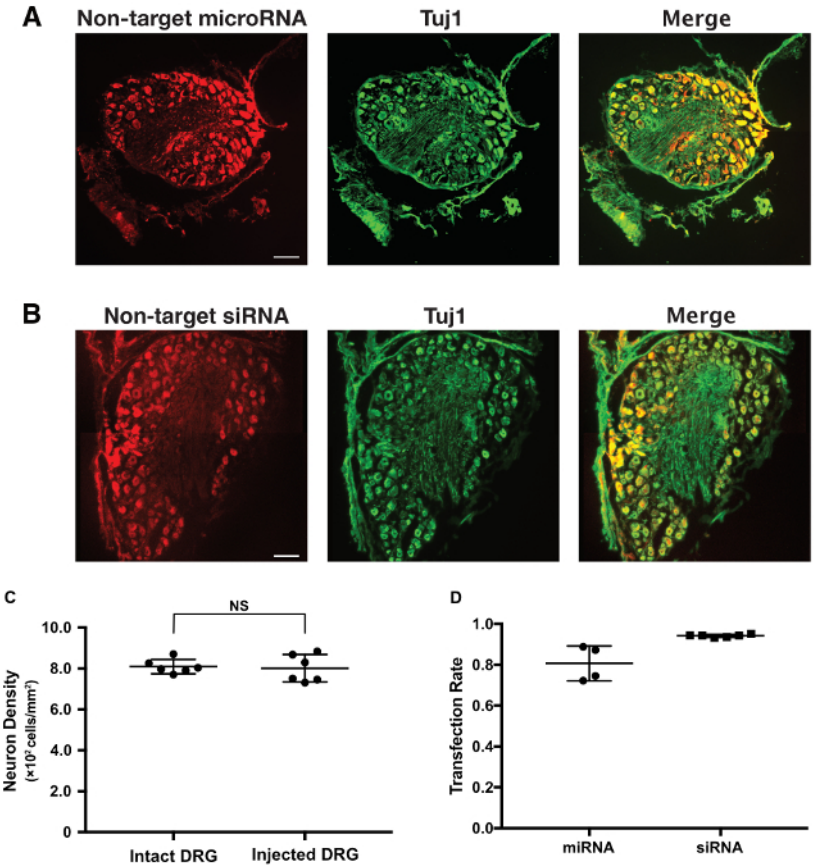


Figure 1: Immunohistochemistry of DRG cryo-section after the electroporation of fluorescently-tagged non-specific microRNA or siRNA *in vivo*. (**A**) Representative cryo-section of DRG tissue injected and electroporated with fluorescently-tagged (Dy547) non-target microRNAs. Left image: the image of the fluorescent signal (red color) of fluorescently-tagged (Dy547) non-target microRNAs. Middle image: immuno-staining (green color) of Tuj1 with an Alexa488 fluorophore on the secondary antibody. Right image: the merged image of the previous two channels. Scale bar = 100 μm.*(***B**) Representative cryo-section of DRG tissue injected and electroporated with fluorescently-tagged (Cy3) non-target siRNAs. Left image: the image of the fluorescent signal (red color) of fluorescently-tagged (Cy3) non-target siRNAs. Middle image: immuno-staining (green color) of Tuj1 with an Alexa488 fluorophore on the secondary antibody. Right image: the merged image of the previous two channels. The scale bar represents 100 μm. (**C**) The neuron density of an intact DRG cryo-section is 809.6 ± 14.2 cells/mm^2 (N = 6) and an injected DRG cryo-section is 801.6 ± 27.4 cells/mm^2 (N = 6), Mean ± SEM, Student's *t*-test, NS: no significance. Three mice were performed *in vivo* DRG electroporation on the left L4 and L5 DRGs with tagged siRNA. Both left and right L4 and L5 DRGs are harvested after 48 h and processed through cryo-section and immunohistochemistry. Each DRG has been sectioned into approximate 60 slices and the thickness of the slice is 10 μm. Three slices of each DRG are selected by 200 μm and averaged. (**D**) The transfection rate of the tagged miRNA is 80.7 ± 4.3% (N = 4) and the tagged siRNA is 94.2 ± 0.3% (N = 6), Mean ± SEM. Three mice were performed *in vivo* DRG electroporation on the left L4 and L5 DRGs with tagged siRNA and two mice with tagged miRNA. The DRGs are harvested after 48 h and processed through cryo-section and immunohistochemistry. Each DRG has been sectioned into approximate 60 slices and the thickness of the slice is 10 μm. Three slices of each DRG are selected by 200 μm and averaged. Please click here to view a larger version of this figure.

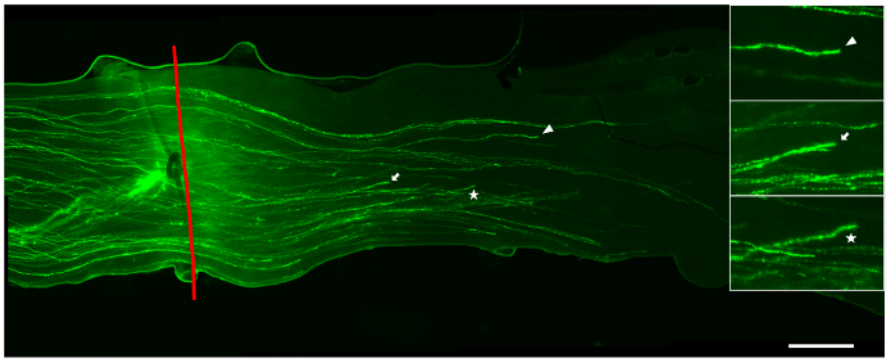


Figure 2: Ectopically expressed EGFP displays regenerated neuronal axons in sciatic nerve after crushing injury. The axons in a flattened sciatic nerve suffered from crush injury are labeled with EGFP (green fluorescence) transported from the somas to axons. The red line indicates the position of the crushing injury site, which was originally surgically marked by a suturing knot. The white arrowhead, arrow, and star indicate three distinctive axon ends, all of which extend from the crush site. Scale bar = 500 µm. Please click here to view a larger version of this figure.

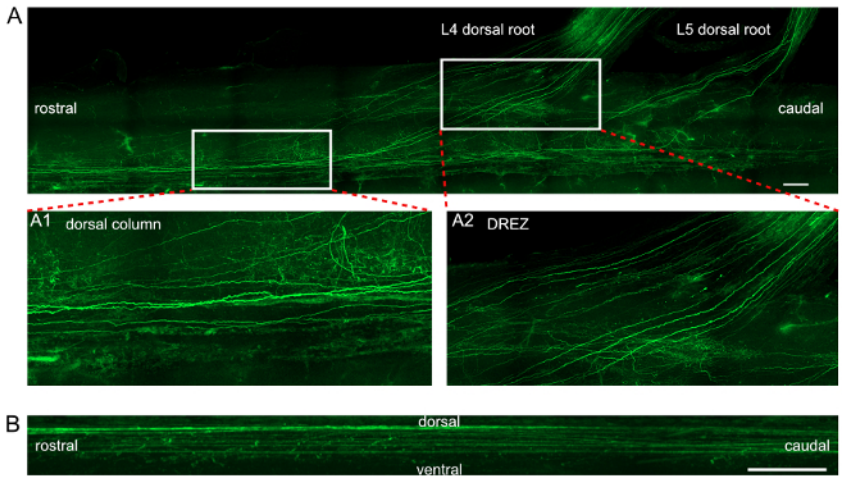
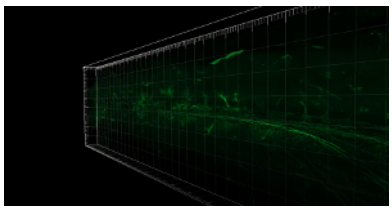


Figure 3: Projection images of EGFP-expressing axons in the spinal cord after *in vivo* electroporation of DRGs. (**A**) Longitudinal projection of the EGFP-labeled axons within the dorsal column and the L4/L5 dorsal roots. Inset images A1 and A2 show magnified views of the two rectangular areas marked in panel (A). A1 exhibits a detailed view of axons in the dorsal column. A2 exhibits a detailed view of axons in the dorsal root entry zone (DREZ). Scale bar = 200 µm. (**B**) Sagittal projection of the EGFP-labeled axons within the dorsal column. Scale bar = 50 µm. Please click here to view a larger version of this figure.



Movie 1: 3D reconstruction of EGFP-expressing axons in the spinal cord after *in vivo* electroporation of DRGs. Please click here to view this video. (Right-click to download.)

Discussion

Several surgical steps require particular attention. The L4 and L5 DRGs (location of somas), which dominate the sciatic nerve, need to be correctly identified and injected with gene constructs. Otherwise, the GFP-labeling will be absent in sciatic nerve axons. The iliac crests can be viewed as useful anatomical landmarks to pinpoint L4 and L5 DRGs. In most mice, the facet joint between L5 and L6 vertebrae is proximate to iliac crests[12]. Alternatively, L3 DRG can be chosen instead of L5, especially for assays such as immunohistochemistry or western blot[13], as surgical exposure of L5 DRG usually meets great difficulties due to a deep location and rich blood supply. Also, the whole surgery should avoid damaging DRG surrounding structures, including the spinal cord and the nerve root. The second critical operational step worth noting is the injection. The Fast Green dye was mixed with the solution of gene constructs to visualize the solution ejected from the needle and diffuse in the DRG. A successful injection should show a clear round-shape DRG outlined by the fluorescent dye. If the solution with fluorescent dye leaks out

from the DRG while injecting, a back-and-forth fine-tuning on the depth of the tip of micro-needle can ensure complete injection of all solution into the DRG capsule. Avoid impaling the DRG at more than three different sites. Further, mild bleeding is usually inevitable after DRG injection, as DRGs are tightly encapsulated with rete venosum[13]. It is important to stop bleeding so that there will not be blood insulating the surface of DRG from electrodes during electroporation. Successful electroporation depends on electric current transduction, and PBS solution can be applied on the electrode. Finally, the site on the sciatic nerve where is crushed with forceps should be marked with micro-suturing, because the injury site is invisible under the microscope and needs to be indicated as the starting point of axon regeneration for image analysis later. If the epineural suture knot at the crush site falls off after animal perfusion and dissection, it is critical to re-label the same site with a suture immediately on the PFA-fixed nerve when the crush-induced indentation is still identifiable.

Among transfection techniques, electroporation has a higher transfection rate. According to our study, the transfection rate of the DRG neuron for microRNAs or siRNAs after *in vivo* electroporation is close to 90%. More importantly, *in vivo* electroporation is far less time-consuming than virus-based methods, which require virus packaging of desired gene constructs. As far as we know, even though the lipofectamine-based *in vivo* transfection has less toxicity than electroporation, the lipofectamine does not work on DRG neurons particularly, neither *in vivo* nor *in vitro*. Noteworthy, the current methodology has several technical limitations. First, the duration of siRNA efficacy to knocking down gene-of-interest is shorter than the virus-based delivery of plasmids. Therefore, the whole procedure from electroporation to animal sacrifice has to be finished in 4–6 days[14]. Additionally, the surgery performed under the microscope requires practice and some manual dexterity. For a novice, the surgery duration is often longer than expected. The anesthetic dose administrated to the mouse has to be carefully controlled to prevent unwanted mortality. Finally, flattening the sciatic nerve causes overlapping of axons when conducting epifluorescent imaging. Imaging the unflattened nerves with a confocal microscope is an alternative option.

Anatomically, the DRG sensory neurons have two axonal branches - the peripheral descending branch and the ascending central branch projecting into the dorsal column of the spinal cord[10]. The current methodology also shows distinctive labeling of the dorsal column of the spinal cord. Thus, a similar methodology can be used as a model to investigate sensory axon regeneration after spinal cord injury. Combined with tissue-clearing techniques[11], conventional confocal microscopy or light-sheet microscopy can be employed on the cleared spinal cord sample to build 3D reconstructed images of sensory axons within the dorsal column of the spinal cord.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The study was funded (awarded to F-Q.Z.) by NIH (R01NS064288, R01NS085176, R01GM111514, R01EY027347), the Craig H. Neilsen Foundation and the BrightFocus Foundation.

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