Published in final edited form as:

J Neurosci Methods. 2016 September 15; 271: 34–42. doi:10.1016/j.jneumeth.2016.05.017.

RetroDISCO: Clearing technique to improve quantification of retrograde labeled motor neurons of intact mouse spinal cords

Emilija Žygelyte^a, Megan E. Bernard^a, Joy E. Tomlinson^a, Matthew J. Martin^a, Allegra Terhorst^a, Harriet E. Bradford^{a,b}, Sarah A. Lundquist^a, Michael Sledziona^{a,b}, and Jonathan Cheetham^{a,*}

^aDepartment of Clinical Sciences, Cornell College of Veterinary Medicine, Cornell University, Ithaca, NY, United States

^bThe Royal Veterinary College, University of London, North Mymms, Hertfordshire, UK

Abstract

Background—Quantification of the number of axons reinnervating a target organ is often used to assess regeneration after peripheral nerve repair, but because of axonal branching, this method can overestimate the number of motor neurons regenerating across an injury. Current methods to count the number of regenerated motor neurons include retrograde labeling followed by cryosectioning and counting labeled motor neuron cell bodies, however, the process of sectioning introduces error from potential double counting of cells in adjacent sections.

New method—We describe a method, retroDISCO, that optically clears whole mouse spinal cord without loss of fluorescent signal to allow imaging of retrograde labeled motor neurons using confocal microscopy.

Results—Complete optical clearing of spinal cords takes four hours and confocal microscopy can obtain z-stacks of labeled motor neuron pools within 3–5 min. The technique is able to detect anticipated differences in motor neuron number after cross-suture and conduit repair compared to intact mice and is highly repeatable.

Comparison with existing method—RetroDISCO is inexpensive, simple, robust and uses commonly available microscopy techniques to determine the number of motor neurons extending axons across an injury site, avoiding the need for labor-intensive cryosectioning and potential double counting of motor neuron cell bodies in adjacent sections.

Conclusions—RetroDISCO allows rapid quantification of the degree of reinnervation without the confounding produced by axonal sprouting.

Keywords

Retrograde tracer; Mouse; Optical clearing; Confocal microscopy; Regeneration

*Corresponding author at: Department of Clinical Sciences, Box 34, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States. Fax: +1 607 253 3271.

Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jneumeth.2016.05.017>.

1. Introduction

Peripheral nerve injuries affect approximately 360,000 people in the United States annually, mostly as a consequence of trauma (Midha, 1997; Noble et al., 1998; Li et al., 2014). These injuries result in sensory and motor impairments and generate substantial health care costs (Taylor et al., 2008). Despite advances in microsurgical and supportive techniques and substantial work to promote nerve regeneration, recovery following nerve repair is often disappointing and full function is seldom achieved (Kouyoumdjian, 2006; Muir, 2010; Mackinnon and Dellon, 1988; Mackinnon et al., 2001; Pfister et al., 2011).

In order to improve functional outcomes in clinical patients, robust techniques for quantifying the effectiveness of regenerative approaches to nerve injury are essential. Outcome measures to assess regeneration in experimental paradigms of nerve repair are generally selected to quantify the speed and extent of axonal outgrowth, the degree of misdirection of regenerating axons and the number of motor neurons (MNs) regenerating across an injury site (Wood et al., 2011).

Retrograde tracing techniques have been used to quantify the number of MNs reinnervating a target muscle, and are commonly used in mouse models of nerve injury which allow for genetic manipulation of factors affecting nerve regeneration (Walker et al., 1994; Demedinaceli et al., 1982). This technique is particularly useful because axon counts, a commonly used parameter to determine regenerative success (Wood et al., 2011; Pfister et al., 2011), can be confounded by axonal branching. A single parent axon in the proximal stump can produce up to 20 axons extending into the distal stump (Aitken et al., 1947; Mackinnon et al., 1991), which can cause overestimation of the number of reinnervated motor units in the target muscle. By combining morphological analysis of the regenerating axons with retrograde tracing, it is possible to quantify the number of motor units and the extent of neuronal sprouting (Streppel et al., 2002; Boyd and Gordon, 2003; Wood et al., 2010, 2009).

Standard handling of fixed retrograde labeled MN pools within the spinal column consists of snap-freezing, cryosectioning at 20–50 μm , imaging, and manual counting of backlabeled cell bodies (Pot et al., 2002; David and Aguayo, 1985; Taylor et al., 1983; Boyd and Gordon, 2002). This is a technically challenging procedure with potential for experimental variation in several steps. Potential pitfalls include: cracking of the sample during snap-freezing, loss of tissue during sectioning, and double counting of cells split between sections. Adjustment for double counting is feasible but can also generate bias (Abercrombie, 1946).

Recently a technique for optical clearing of intact nervous tissue has been developed which allows three-dimensional imaging of solvent-cleared organs (3DISCO) using ultramicroscopy (Erturk et al., 2012). This approach has been particularly useful for tracing neuronal tracts within the brain. CNS tissue is dehydrated using increasing concentrations of tetrahydrofuran (THF) and subsequently immersed in dibenzyl ether (DBE) which has the same refractive index as the tissue in question (Erturk et al., 2012). This renders the sample transparent and able to be imaged in its entirety without the need for histological sectioning.

One advantage of three dimensional data sets is that they allow for increased flexibility in quantifying spatial relationships between structures that would otherwise lie in multiple two-dimensional planes.

Here we describe the validation and application of a simple, robust, and inexpensive clearing technique to image backlabeled MN pools within the intact spinal cord of mice using confocal microscopy. We then use this new technique to examine the changes in MN number and density after two types of nerve repair and with age in mice. We compare data obtained using this technique to values obtained from the traditional cryosectioning technique.

2. Materials and methods

Intact MN pools were imaged using confocal microscopy in cleared, intact spinal cords following retrograde labeling in five experimental groups of C57BL/6J mice (Jackson): (1) intact sciatic nerves of 1.5 months of age (young) mice, (2) intact sciatic nerves of 10 months of age (old) mice, (3) injured sciatic nerve 8 weeks after creation of a 3 mm sciatic nerve gap repaired with an inert conduit in young mice, (4) intact common peroneal (CP) nerve in young mice, and (5) injured CP nerve 12 weeks after common peroneal to tibial (CP-TIB) cross-suture in young mice. Two retrograde tracers were evaluated, FluoroRuby (Life Sciences) and FastBlue (Polysciences).

This study was performed in accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the NIH guide for Care and Use of Laboratory Animals, federal and state regulations, and was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC). ARRIVE guidelines for reporting *in vivo* experiments were used throughout (Kilkenny et al., 2010).

Animals were allowed to acclimatize for three days after being brought into the research unit prior to any procedure. Daily record logs of medical procedures were maintained. Rodent cages were replaced weekly. Animals were on a 12/12 h light–dark cycle and allowed food and water *ad libitum*. Group housing prior to medical procedures provided socialization.

Mice were chosen for this study because of their common use in nerve injury studies and the ability to selectively manipulate their genome to investigate mechanistic aspects of nerve repair, as well as ease of access and care (Wood et al., 2011).

2.1. Retrograde labeling

Mice were anesthetized and maintained under anesthesia with isoflurane/oxygen. The nerve of interest was exposed and transected. The proximal nerve stump was immersed in a well filled with retrograde tracer (10% FluoroRuby in 2% DMSO or 3% FastBlue in 2% DMSO), the top of the well was sealed with Vaseline, and the nerve was allowed to soak for 1 h (Midha et al., 2005; Al-Majed et al., 2000). The labeled stump was then lavaged with saline and muscular and skin layers closed routinely. Analgesia was provided by subcutaneous meloxicam (2 mg/kg) preoperatively and 12 h after surgery. Animals were allowed to recover for five days to allow for endocytosis and retrograde transport of the tracer.

2.2. Uninjured nerve

To compare neuronal pools from young and old mice, intact sciatic nerves of C57BL/6J females ($n = 9$, 1.5 months of age, 17 ± 2 g; $n = 9$, 10 months of age, 31 ± 2.5 g) were retrograde labeled. The left sciatic nerve trunk was exposed and transected approximately 1 mm proximal to the trifurcation and the proximal stump was labeled. As a control for the cross-suture injury group, the CP nerves of young mice ($n = 6$, female C57BL/6J, 1.5 months) were labeled with FluoroRuby. The CP nerve was identified and transected 3 mm distal to the bifurcation.

2.3. Nerve injury

To test the ability of the clearing technique to determine the number of axons crossing an injury site, two experimental models were used. In the first, a non-critical defect (3 mm) was created by transecting the sciatic nerve and suturing the proximal and distal stumps 1 mm into a 5 mm silicone conduit using 10-0 ethilon suture (Ethicon). Mice ($n = 6$, female C57BL/6J, 1.5 months) were allowed to recover for 8 weeks before application of retrograde tracer. CP-TIB cross-suture performed immediately after transection using 10-0 ethilon suture was used as a model of a nerve graft (Fu and Gordon, 1995b, 1995a). Mice ($n = 6$, female C57BL/6J, 1.5 months) were allowed to recover for 12 weeks before application of retrograde tracer.

2.4. Spinal cord harvest

Five days after labeling, mice were anaesthetized and perfused with chilled saline followed by 4% paraformaldehyde (Sigma) (Midha et al., 2005; Al-Majed et al., 2000). Following perfusion, intact spinal cords were removed 5 mm cranial and 5 mm caudal to the L4-S3 region to ensure the entire MN cell body pool was harvested. Explanted cords were fixed in 4% paraformaldehyde overnight.

2.5. Optical clearing

The following reagents were prepared prior to clearing: 50%, 70%, 80% THF in distilled water, 100% THF, 100% dichloromethane (DCM), and 100% DBE(Sigma–Aldrich).

Fixed, intact spinal cords were immersed in 50% THF in 3 mL glass vials (Butler). Each vial was wrapped in aluminum foil to avoid photobleaching, placed in a secondary container (15 mL conical tube) to catch any leaks, and rotated at 20–30 rpm for 30 min. The cord was exposed to increasing concentrations of THF (70, 80 and 100%) for 30 min at each step. The 100% THF immersion was repeated three times with fresh 100% THF. Cords were then immersed and rotated in 100% DCM for 20 min followed by 100% DBE for a minimum of 30 min (Table 1).

Following exposure to DBE, spinal cords were placed into a well composed of two FastWell™ reagent barriers (Grace Bio-Labs) adhered on top of each other to a Superfrost® Plus slide (Fisher). Spinal cords were placed within the well dorsal side down, covered with 100% DBE and sealed in with a glass coverslip (Fig. 1). The MN pool of interest is located on the ventral horn of the spinal cord, and correct orientation of the spine on the slide is necessary to obtain a clear image.

To assess any potential decrease in tissue size due to the clearing procedure (Erturk et al., 2012) the length, width, and thickness of the spinal cords were measured before and immediately after clearing using a digital caliper.

2.6. Imaging

Spinal cords mounted in DBE were imaged at 10× magnification with 6.4 µm z-stacks using a confocal microscope (Zeiss 510, Thornwood, NY; 561 nm for Fluoro Ruby, and 405 nm for FastBlue). The confocal microscope was set at a bit depth of 12, averaging of 2, laser intensity 8, 1.0 airy units pinhole, and 600 gain. Adjustments were occasionally made to the gain in order to obtain a brighter image. To test the persistence of the fluorescence associated with FluoroRuby and FastBlue, samples were stored at 4°C away from light, in fastwell slides filled with DBE, and serially imaged for up to 14 days after clearing.

2.7. Counting and analysis

ImageJ software (NIH) was used to identify and manually count cell bodies. Two trained observers, blinded to experimental group, independently counted labeled cell bodies in all samples ($n = 30$). In order to measure cell density, the xy plane area and z plane depth of each cell body pool was determined and volumes calculated. The repeatability of this approach was assessed using standard guidelines (Bland and Altman, 1986). To compare MN counts obtained using retroDISCO with raw and Abercrombie corrected counts (Abercrombie, 1946) obtained on cryosection sample, orthogonal diameters of 75 labelled cell bodies were determined using ImageJ in 22 sections obtained from three mice.

2.8. Cryosectioning

The left sciatic nerves of female C57BL/6J mice ($n = 5$, 1.5 months of age) were retrograde labeled and mice were perfused five days later. Harvested spinal cords were cryoprotected in 4% paraformaldehyde and 30% sucrose overnight and then flash-frozen in liquid nitrogen. Frozen blocks were stored at -80°C overnight. Samples were cryosectioned at 30 µm thickness following standard guidelines (Pot et al., 2002; Baulac and Meininger, 1983) and each section imaged and MNs counted as before.

2.9. Time course

To evaluate the ability of retroDISCO to evaluate regeneration over time, sciatic nerve transection was performed and immediately repaired using a 5 mm silicone conduit (Tuzic, Siliclear tubing, 1.98 mm ID) in young C57/BJ mice. Conduits were left empty or filled with 0.7% agarose (Seaprep, Rockland, ME) prepared by mixing 1.4% agarose solution in DI water then autoclaving to yield 0.7% agarose gel (Balgude et al., 2001). Sterilized conduits were filled with liquid agarose and incubated at 4°C for 15 min to allow gelation before surgical implantation. Retrograde labelling was applied at 2, 4, 8 or 12 weeks after repair ($n = 3-6$ /time point/group) followed by reperfusion five days later. Retrograde labelled motor neuron were manually counted in ImageJ.

2.10. Data analysis

Continuous outcome measures were assessed using *t*-test or ANOVA with Tukey's *post hoc* tests. All data were analyzed using JMP 10 (SAS Institute Inc., Cary, NC). Significance was set as $p < 0.05$ throughout.

3. Results

All spinal cords were rendered translucent by the clearing process (Fig. 2A) and retrograde labeled MN cell bodies could be clearly imaged and counted (Fig. 2B and C and Supplemental video 1). Cell bodies appeared as bright red, stellate areas with extending dendrites. Nuclei were readily identifiable as darker areas within the labeled MNs. As anticipated all sciatic MN cell bodies were concentrated in a single, longitudinal pool, with the highest density centrally, and tapering out towards the cranial and caudal ends.

3.1. Degradation of retrograde tracer

There was no significant decrease in the number of FluoroRuby backlabeled MN cell bodies over the 14 day observation period (Fig. 3). In contrast, the observed number of FastBlue labeled MNs immediately following clearing was significantly lower than those labeled using FluoroRuby ($p < 0.001$), and the signal degraded rapidly, with the observed number of MNs approaching zero by 72 h.

3.2. Repeatability

No evidence of bias was observed in MN counts determined by two independent, blinded observers (Fig. 4). No evidence of a trend towards bias was observed over the range of MN counts obtained ($p = 0.65$). The coefficient of repeatability (CR) was low (CR = 67), suggesting a highly repeatable protocol.

3.3. Cryosectioning

MN diameters obtained from cryosectioned samples were $24.4 \pm 0.5 \mu\text{m}$. As anticipated, MN count estimates were lower in spines cryosectioned at $30 \mu\text{m}$ following Abercrombie correction ($p < 0.0001$) (Fig. 5). RetroDISCO estimates were between corrected and uncorrected estimates for cryosectioned spines obtained from mice of the same age. Attempts to cryosection optically cleared spinal cords, to explore the possibility of colabeling using immunohistochemistry, resulted in fracturing of cleared tissue and major tissue damage that precluded further evaluation of this approach.

3.4. Tissue shrinking

The clearing procedure reduced the volume of mouse spinal cords by approximately 50% (mean $\pm$ se, before clearing $95.1 \pm 9.2 \text{ mm}^3$; post clearing $43.5 \pm 6.5 \text{ mm}^3$, ($p < 0.001$, Fig. 6)).

3.5. Motor neuron pool volume

As anticipated, MN pool volumes of intact CP nerves in young mice (mean $\pm$ se $0.086 \pm 0.011 \text{ mm}^3$) were significantly smaller than those of intact sciatic MN pools in young ($0.12 \pm 0.008 \text{ mm}^3$) and old ($0.16 \pm 0.007 \text{ mm}^3$) mice ($p < 0.001$, Fig. 7).

3.6. Number and density

As anticipated, significant differences in the number of retrograde labeled MN were observed between uninjured mice and mice that had undergone nerve injury and repair by either CP-TIB cross-suture or a conduit placed with a non-critical length sciatic gap (Figs. 8 and 9). There were no significant differences in MN number between young (mean $\pm$ se, 802.4 ± 41.4) and old (771.7 ± 41.4) mice. MN counts in animals with a sciatic gap injury were less than half those of intact mice (298 ± 50.7 ; $p < 0.0001$). MN counts for mice who had undergone a CP-TIB cross suture were significantly lower than mice with intact CP nerves (mean $\pm$ se $311.5.6 \pm 29.8$ for injured mice; 438.6 ± 32.6 for uninjured mice; $p = 0.018$).

As anticipated, MN pool volumes of intact CP nerves in young mice (mean $\pm$ se $0.086 \pm 0.011 \text{ mm}^3$) were significantly smaller than those of intact sciatic MN pools in young ($0.12 \pm 0.008 \text{ mm}^3$) and old ($0.16 \pm 0.007 \text{ mm}^3$) mice ($p < 0.001$, Fig. 7). Mice with CP-TIB cross suture had a significantly lower MN density ($3168 \pm 428 \text{ MN/mm}^3$) than mice with an intact CP nerve ($5324 \pm 479 \text{ MN/mm}^3$, $p = 0.012$, Figs. 8 and 9).

3.7. Time course

Increasing numbers of retrograde labelled motor neurons were identified at four and eight weeks after repair compared with two weeks (Fig. 10, $p < 0.001$). No significant differences were noted between agarose filled and empty conduits at any time point (all $p > 0.05$).

4. Discussion

4.1. Feasibility and repeatability

Clearing of intact spinal cords using THF/DBE was technically straight forward, and produced high resolution confocal z-stacks with readily identifiable back labeled MN cell bodies. The clarity of the images obtained enabled highly repeatable quantification of MN number by two independent observers. Imaging of cleared spines using confocal microscopy allows widely accessible, rapid, repeatable counts of labeled MNs. Motor neuron counts for uninjured sciatic nerve were comparable to motor neuron counts obtained by others (Baulac and Meininger, 1983). Additionally, applying the Abercrombie method to MN counts from cryosections would decrease the actual cell count by approximately 10%, bringing down the number of motor neurons counted when using cryosectioning much closer to the number obtained with clearing (Smolen et al., 1983).

The fluorescent tracer, FluoroRuby is a nontoxic and inert hydrophilic polysaccharide retrograde tracer commonly used for MN counting (Hayashi et al., 2007). FluoroRuby produces a deep red fluorescence (excitation maximum: 555 nm, emission: 580 nm) and labels the cytoplasm and proximal dendrites (Schmued et al., 1990). FluoroRuby signal was

retained over the 14 day observation period with a minimal decrease in the observed number of labeled cell bodies.

One limitation of FluoroRuby is that uptake might be restricted when it is applied by intramuscular injection thus FluoroRuby application is limited to transected nerves (Richmond et al., 1994; Puigdemívol-Sánchez et al., 2000).

A further limitation is that *in vivo*, FluoroRuby fades more rapidly than other tracers such as FastBlue or FluoroGold and so is only suitable for short term labeling of less than four weeks as described here (Novikova et al., 1997; Choi et al., 2002). Use of the reservoir technique for FluoroRuby application and spinal cord fixation five days following application was effective in these experiments as anticipated and previously described (Novikova et al., 1997; Madison et al., 1996; Harsh et al., 1991).

FastBlue is described as an intensely fluorescent retrograde tracer with strong *in vivo* persistence and stability (Puigdemívol-Sánchez et al., 2002; Novikova et al., 1997). When exposed to THF/DBE, the fluorescent signal faded very rapidly. MN counts immediately after the clearing procedure were 45% lower in Fast-Blue labeled cell bodies as compared to counts obtained using FluoroRuby. These data suggest that FastBlue should not be used in conjunction with the tissue clearing technique described here. The clearing procedure degrades lipophilic dyes, thus FluoroGold, another tracer commonly used for assessing nerve regeneration, is likely not suitable for use in combination with this approach (Ertürk et al., 2012). Further work should elucidate other retrograde tracers that are suitable for use in conjunction with tissue clearing for multiple or sequential labeling experiments.

One major difficulty in the use of retrograde tracers to determine the number of reinnervating MNs after nerve repair has been the likelihood of counting the same cell body multiple times (Clarke, 1992). This problem arises despite efforts to count only MN in which a distinct nucleus can be identified (Novikova et al., 1997; Abercrombie, 1946; Choi et al., 2002). We identified a similar pattern in this study where the observed number of labeled cell bodies from cryosectioned (30 µm) tissue was significantly higher than from cleared, intact tissue. Abercrombie correction reduced the estimate of motor neuron number below that identified by retroDISCO. A major advantage of the clearing technique described here is that by clearly identifying all labeled MN cell bodies in three-dimensions in a single image file (z-stack), the possibility of double-counting is eliminated. In this way, the need for the variety of approaches used to correct for double counting is avoided (Abercrombie, 1946; Smolen et al., 1983; Williams and Rakic, 1988).

RetroDISCO enabled detection of progressive regeneration between two and eight weeks after injury, with no significant difference between the four and eight week time points. We did not observe any significant differences in MN number between sciatic nerves repaired with 0.7% agarose hydrogel and unfilled (empty) conduits. We selected an agarose hydrogel, a biocompatible copolymer that cross-links in a temperature-dependent fashion and reduces scar tissue formation following nerve injury (Dillon et al., 1998), as it could be used as a delivery vehicle for growth factors or cytokines to manipulate the microenvironment at the site of repair (Mokarram et al., 2012).

4.2. Application for evaluation of age-related neuropathies

In this study, no difference in absolute cell body counts between young (1.5 months) and old (10 months) mice was identified. This finding is consistent with previous work suggesting that little change occurs in the sciatic MN counts of mice between two and three months of age (Baulac and Meininger, 1983) and between six and twenty-five months of age in the facial nucleus (Sturrock, 1988). The volume of the sciatic MN cell body pool for 10 month old mice was larger than that for 6 week old mice, resulting in a significantly lower cell density. This is also consistent with earlier work (Baulac and Meininger, 1983).

Understanding how age affects MN numbers is important in the study of age-related declines in neuromuscular function. In general, MN counts and density decrease in the early post natal period (Baulac and Meininger, 1983), and no further significant change is observed in MN counts until 24 months of age after which counts began to decrease (Sturrock 1988; Tanaka and Webster, 1991). Precise estimates of MN number using the clearing technique described here will allow determination of differences between experimental groups in future work.

4.3. Injury

This application was able to detect a difference in MN number between intact nerve and 2 conditions of peripheral nerve injury and 2 durations of regeneration. The 2 timepoints chosen represent expected submaximal and maximal repair. This demonstrates the broad application of the clearing technique to assess regeneration after peripheral nerve repair in a range of commonly used experimental models.

4.4. Limitations

While the clearing procedure was straightforward, some preliminary work was needed to optimize imaging and counting consistency. The greatest gains in this preliminary phase were in image quality, which increased the contrast between labeled MN and background fluorescence.

Animals with uninjured nerves had a large number of cell bodies present within the spinal cord, leading to some overlapping cell bodies in the final image. As the number and density of MN cell bodies increased, distinguishing between individual cells in serial z-layers became more difficult. The possibility that multiple overlapping cells were sometimes counted as one exists, resulting in a modest underrepresentation of the final number of MNs counted. Because of the lower MN density, this phenomenon was not observed in the repaired animals.

A number of methods for imaging whole, cleared tissues have recently emerged. Methods for clearing neural tissue include 3DISCO, iDISCO, CLARITY, and CUBIC (Renier et al., 2014; Erturk et al., 2012; Chung et al., 2013; Susaki et al., 2014). 3DISCO and iDISCO have been used in combination with immunolabeling (Renier et al., 2014). As the retroDISCO method is based upon DBE clearing we anticipate that immunolabeling in combination with retroDISCO would be feasible. This is in contrast to ScaleA2 and CLARITY methods which may have limited compatibility with immunostaining due to the

use of urea and SDS, to which some antigens may be sensitive, in their respective protocols. The entire clearing procedure for retroDISCO could be accomplished in under four hours, as for 3DISCO, and compared to several weeks for CLARITY or CUBIC (Erturk et al., 2012). In addition, retroDISCO was simple to learn and all chemicals were readily attainable.

5. Conclusions

Retrograde labeling of axons distal to a site of repair using fluorescent dyes is a fundamental approach for demonstrating the connectivity of the peripheral nerve to the spinal cord and determining the degree of regeneration. RetroDISCO, described here, allows clearing of intact mouse spinal cord for imaging of retrograde labeled cells *via* confocal microscopy and is both technically feasible and offers multiple advantages over the current standard sectioning and imaging techniques. This approach is highly repeatable, less labor intensive and more rapid than existing techniques. The clearing approach also preserves the spatial relationships between MNs, opening the possibility of future mapping studies using multiple labels. Confocal microscopy is commonly available to researchers and so this approach should be widely accessible. This technique can be used in combination with a wide variety of experimental paradigms to answer key outstanding questions in peripheral nerve repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partially supported by the National Institute on Deafness and Communication Disorders of the National Institutes of Health under award number RO3DC013376

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Highlights

- RetroDISCO optically clears whole mouse spinal cord while retaining fluorescent signal.
- The technique is tracer dependent and detects expected differences after repair.
- RetroDISCO is inexpensive, simple, robust and uses confocal microscopy.

Fig. 1.

After the clearing procedure, spinal cords (A) were placed dorsal side down in a well consisting of two stacked FastWell™ (B) barriers adhered to a microscope slide (C), covered with 100% DBE, and sealed with a glass coverslip (D).

Fig. 2.

RetroDISCO renders spinal cords transparent and allows imaging of labeled motor neurons. A) An intact mouse spinal cord (a) before and (b) after the clearing procedure. B) Rendering the spinal cord transparent allowed confocal imaging of sciatic motor neurons, located in the dorsal aspect of the ventral horn in the L4-S3 region. C) Cell bodies appeared as bright red, stellate areas with extending dendrites. Nuclei were identifiable as darker areas within the labeled motor neuron cell bodies.

Fig. 3. Relative persistence of fluorescence for FluoroRuby and FastBlue in serially imaged mouse spinal cords after clearing. A) FluoroRuby fluorescence persisted over the 14 day observation period ($n = 6$) while a rapid decrease in fluorescence was observed for FastBlue ($n = 6$). Data shown are mean $\pm$ standard error. B) Spinal cords labeled with FluoroRuby were imaged immediately after clearing, at 24 hours, and at 14 days with minimal loss of fluorescence observed. C) Spinal cords labeled with FastBlue were imaged immediately after clearing, after 24 hours, and after three days. A substantial decrease in fluorescence was observed after 24 hours, and no labelled cell bodies were visible three days after the clearing procedure.

Fig. 4.

Bland-Altman plot of motor neuron counts obtained by two independent observers ($n = 30$). No evidence of a trend towards bias was observed over the range of motor neuron counts obtained ($p = 0.65$). Dotted lines are 95% CI. The coefficient of repeatability (CR) was low (CR = 67), suggesting a highly repeatable protocol.

Fig. 5. Motor neuron numbers obtained from sciatic nerves back labeled with FluoroRuby using clearing of intact spines ($n = 9$) conventional cryosectioning ($n = 6$) or conventional cryosectioning with Abercrombie correction ($n = 6$). Estimates of MN number were higher in samples cryosectioned at 30 μm without Abercrombie correction and than in cleared spinal cords (retroDISCO) from mice of the same age ($p = 0.002$). Letters denote significant differences between groups (Tukey's *post hoc* test).

Fig. 6. RetroDisco reduces the volume of the spinal cord by approximately 50% in both young (***, $p = 0.0003$) and old mice. Mean $\pm$ SEM data shown pre and post clearing.

Fig. 7.

Motor neuron pool volumes in intact common peroneal (CP) nerves of young mice were significantly lower than those of young and old mice with intact sciatic nerves ($p < 0.001$). Letters denote significant differences between groups (Tukey's *post hoc* test).

Fig. 8.

Number and density of retrograde labeled MN in mice 8 weeks after creation of a 3 mm sciatic nerve gap (conduit), and intact sciatic nerve of 6-week (young) and 10-month old (old) mice. Significant differences in motor neuron number were not observed between young and old mice, but counts in mice with a conduit repair were significantly lower ($p < 0.0001$). The density of cell bodies within the motor neuron pool was significantly different between conduit graft, young and old mice ($p < 0.0001$). Letters denote significant differences between groups (Tukey's *post hoc* test).

Fig. 9.

Number and density of retrograde labeled motor neurons in mice 12 weeks after common peroneal to tibial cross-suture and mice with an intact common peroneal nerve. Motor neuron counts for mice who had undergone a CP-TIB cross suture were significantly lower than mice with intact CP nerves ($p = 0.018$). Mice with a CP-TIB cross suture had a significantly lower motor neuron density than mice with an intact common peroneal nerve ($5324 \pm 79 \text{ MN/mm}^3$, $p = 0.012$).

Fig. 10.

Progressive increase in numbers of retrograde labelled motor units neurons over time ($p < 0.001$). No significant differences were noted between sciatic nerve repair with a 5 mm conduit filled with 0.7% agarose or an empty conduit at any time point (all $p > 0.05$). Letters denote significant differences between groups, $n = 4-6$ mice/group/time point.

Table 1

Summary of the clearing process for retrograde labeled spinal cords.

Clearing agent	Time	Number of washes
50% THF	30 minutes	1
70% THF	30 minutes	1
80% THF	30 minutes	1
100% THF	30 minutes	3
100% DCM	20 minutes	1
100% DBE	>30 minutes	1