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Semi-automated counting of axon regeneration in poly(lactide *co*-glycolide) spinal cord bridges

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HIGHLIGHTS

- Hessian-based filtering improves the image contrast of spinal cord axon staining.
- Axons can be detected within the filtered images with accurate, automated counting.
- Filtering and semi-automated counting effectively estimates the number of myelinated axons.

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ABSTRACT

Background: Spinal cord injury (SCI) is a debilitating event with multiple mechanisms of degeneration leading to life-long paralysis. Biomaterial strategies, including bridges that span the injury and provide a pathway to reconnect severed regions of the spinal cord, can promote partial restoration of motor function following SCI. Axon growth through the bridge is essential to characterizing regeneration, as recovery can occur via other mechanisms such as plasticity. Quantitative analysis of axons by manual counting of histological sections can be slow, which can limit the number of bridge designs evaluated. In this study, we report a semi-automated process to resolve axon numbers in histological sections, which allows for efficient analysis of large data sets.
New method: Axon numbers were estimated in SCI cross-sections from animals implanted with poly(lactide *co*-glycolide) (PLG) bridges with multiple channels for guiding axons. Immunofluorescence images of histological sections were filtered using a Hessian-based approach prior to threshold detection to improve the signal-to-noise ratio and filter out background staining associated with PLG polymer.
Results: Semi-automated counting successfully recapitulated average axon densities and myelination in a blinded PLG bridge implantation study.
Comparison with existing methods: Axon counts obtained with the semi-automated technique correlated well with manual axon counts from blinded independent observers across sections with a wide range of total axons.
Conclusions: This semi-automated detection of Hessian-filtered axons provides an accurate and significantly faster alternative to manual counting of axons for quantitative analysis of regeneration following SCI.

1. Introduction

Spinal cord injury (SCI) is a traumatic event that affects approximately 12,000 new individuals in the United States each year (Devivo, 2012; National Spinal Cord Injury Statistical, 2013). Ascending and descending axon tracts are disrupted during SCI resulting in loss of sensory and motor function (McDonald and

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Sadowsky, 2002). Many pre-clinical strategies, including peripheral nerve and cell grafts, have been developed to reconnect severed regions of the cord after SCI (Cheng et al., 1996; David and Aguayo, 1981; Lu et al., 2012; Thuret et al., 2006). Biomaterial bridges have been shown to guide axons across the injury site (Hurtado et al., 2006; King et al., 2003; Moore et al., 2006; Novikov et al., 2002; Pawar et al., 2015; Stokols et al., 2006; Thomas et al., 2013; Tuinstra et al., 2014; Wong et al., 2008) and provide modest improvement in locomotion when coupled with cell transplantation or growth factor delivery (Han et al., 2009; Teng et al., 2002; Tsai et al., 2006). The extent of regeneration through the bridge, however, is often not characterized and recovery may be due to indirect mechanisms including modulation of the immune response, spinal plasticity, and altered cell migration. To examine the role of regeneration in recovery, and to compare different studies, quantitative analysis of axon regrowth through the injury site is needed.

Manual counting of axons in serial sections through the injury site can be time-intensive. Assessing myelination of regenerating fibers requires additional counting and coordination of multi-color images to determine co-localization of axonal and myelin staining. The time necessary to obtain these data can hinder larger studies, such as combinatorial strategies where several factors are tested simultaneously. Automated image analysis has the potential to expedite this process and allow for large, multi-variable studies. Few automated techniques, however, have been developed for quantification of axons or myelination of regenerating axons in the injured spinal cord. Axon densities have been estimated using line profiles, though only features that intersect with the line profile region are analyzed (Grider et al., 2006; Sathyanesan et al., 2012). Variation in axon density across two dimensions in a section cannot be well captured. Regeneration following SCI is often inhomogeneous, with regions of high and low axon density within the same tissue section, requiring methods to approximate total axon numbers rather than densities of axons. Another major obstacle to automated analysis of regeneration is inconsistency in fluorescence due to non-specific background staining and autofluorescence from implanted materials, such as bridges, that limits the use of simple threshold techniques for object detection and counting.

In this study, we describe a semi-automated approach to estimate axon numbers and numbers of remyelinated axons that is consistent with counts performed by blinded observers, yet can be performed in significantly less time. This analysis is applied to quantify axon growth through PLG spinal cord bridges that contain multiple channels to promote directed regrowth through a lateral hemisection model of spinal cord injury (Thomas et al., 2013; Tuinstra et al., 2014; Yang et al., 2009). Histological sections were stained for neurofilament, and images were captured and filtered using a Hessian-based technique to overcome low signal-to-noise ratios. Importantly, the eigenvalues of the Hessian matrix are used to detect curvilinear structures within the image. Similar techniques have been developed to improve the contrast of blood vessels in magnetic resonance imaging for two- and three-dimensional morphological analysis (Frangi et al., 1998; Sato et al., 1998). Hessian-based filtering followed by object detection provides a time-saving tool for semi-automated analysis of axon regeneration and myelination following SCI.

2. Methods

2.1. PLG bridge scaffolds

Porous multiple channel bridges were fabricated from PLG (75:25 ratio of D,L-lactide to L-glycolide; inherent viscosity: 0.76 dL g^{-1}; Lakeshore Biomaterials; Birmingham, AL, USA) and 63–106 μm salt as previously described (Thomas et al., 2013). Briefly, sugar fibers were drawn from 220 °C caramelized blend of dextran (MW ~100,000; Sigma Aldrich; St. Louis, MO, USA), glucose (Sigma Aldrich), and sucrose (Fisher Scientific; Pittsburgh, PA, USA) in a 1/2.5/5.3 ratio by mass. Seven fibers were dipped into a 2.5:1 mixture by mass of PLG microspheres to salt, packed into a custom mold, and then equilibrated under 800 psi CO_2 for 16 h. The CO_2 was released over 30 min, foaming the bridge into its final structure. The bridges were then cut to 2 mm in length, leached for 4 h in ultrapure water, dried, and stored in a desiccator until use. Prior to implantation, bridges were immersed in 70% ethanol and then ultrapure water for 30 s each.

2.2. Spinal cord injuries

Female C57Bl/6 mice (age 8–10 weeks) were used for spinal cord injuries studies in accordance with the ACUC at Northwestern University. Animals were anaesthetized using 2% isoflurane and the surgery site was scrubbed by repeated betadine and ethanol exposure. A single incision was made in the skin to expose the underlying muscle. The muscle tissue next to the spinal column was cut from T7–T11. A double laminectomy was performed from T9–T10. Two lateral hemisection incisions were created between T9 and T10, approximately 2 mm apart. The tissue between each incision was cleared to create a gap hemisection injury. A PLG spinal cord bridge was then implanted into the injury site and covered with gel foam (Henry Schein; Dublin, OH, USA). The musculature was then sutured closed and the skin closed using surgical clips. Animals were expressed twice daily and given enrofloxacin (Baytril, Henry Schein, 2.5 mg/kg per day) for two weeks following injury. For analgesia, buprenorphine (0.1 mg/kg) was administered every 12 h subcutaneously for 60 h following surgical procedures.

2.3. Tissue harvest and neurofilament labeling

At 8 weeks post injury, animals were perfused and the spinal cord tissue from T7 to T12 was harvested. Spinal cords were embedded and 12 μm cryostat sections were obtained in the cross-sectional plane. Sections were batch stained for each analysis. Sections were post-fixed for 10 min in 4% paraformaldehyde (Sigma) followed by permeabilization in 0.5% triton-X (Sigma) in Trizma solution (Sigma) for 15 min. Sections were then blocked in 10% normal donkey serum (Jackson Immuno Research; West Grove, PA, USA) and 5% bovine serum albumin (Sigma) in 0.1% triton in Trizma solution for 1 h. Sections were incubated with the following primary antibodies overnight: rabbit anti-neurofilament (NF200, 1:200, Sigma) and goat anti-myelin basic protein (MBP; 1:500; Santa Cruz, Dallas, TX, USA). Sections were washed 3 times, incubated with the donkey anti-rabbit Alexa Fluor 555 and donkey anti-goat Alexa Fluor 647 secondary antibodies (1:1000; Life Technologies, Grand Island, NY, USA) for 1 h, and then mounted with coverslips using Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA). A series of 200× images encompassing the entire cross-section were obtained for NF200 and MBP stains on a widefield fluorescent microscope (DMIRB; Leica, Buffalo Grove, IL, USA) with standard fluorescence filter cube sets, a 0.4 numerical aperture 20x objective, and a 14-bit CCD camera (CoolSnap HQ-2; Photometrics, Tucson, AZ, USA) resulting in an approximate pixel size of 0.67 μm^2. Images for each stain corresponding to the same 200x region were converted to 8-bit and merged first prior to stitching. Merged images from the same spinal cord cross-section were then stitched in Photoshop (Adobe, San Jose, CA, USA), resulting in a single composite image of NF200 and MBP staining in the entire section. All sections were stained and imaged under the same conditions.