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A semi-automated method for identifying and measuring myelinated nerve fibers in scanning electron microscope images

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ABSTRACT

Diagnosing illnesses, developing and comparing treatment methods, and conducting research on the organization of the peripheral nervous system often require the analysis of peripheral nerve images to quantify the number, myelination, and size of axons in a nerve. Current methods that require manually labeling each axon can be extremely time-consuming as a single nerve can contain thousands of axons. To improve efficiency, we developed a computer-assisted axon identification and analysis method that is capable of analyzing and measuring sub-images covering the nerve cross-section, acquired using a scanning electron microscope. This algorithm performs three main procedures – it first uses cross-correlation to combine the acquired sub-images into a large image showing the entire nerve cross-section, then identifies and individually labels axons using a series of image intensity and shape criteria, and finally identifies and labels the myelin sheath of each axon using a region growing algorithm with the geometric centers of axons as seeds. To ensure accurate analysis of the image, we incorporated manual supervision to remove mislabeled axons and add missed axons. The typical user-assisted processing time for a two-megapixel image containing over 2000 axons was less than 1 h. This speed was almost eight times faster than the time required to manually process the same image. Our method has proven to be well suited for identifying axons and their characteristics, and represents a significant time savings over traditional manual methods.

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1. Introduction

Peripheral nerves, such as the sciatic nerve in the leg, are responsible for the essential task of conducting information about sensation and movement between the central nervous system and the rest of the body. Consequently, there are hundreds of studies investigating the structure and function of these nerves, their reaction to injury, and mechanisms of regrowth. Many of these studies rely on measurements of nerve characteristics such as the number and size of nerve cell projections known as axons (e.g. Gasser and Grundfest, 1939; Hursh, 1939; Edds, 1950; Fried and Hildebrand, 1982; Knox et al., 1989; Wattig et al., 1992; Sullivan et al., 2003; Demirer et al., 2006). In vertebrates, an insulating sheath known as myelin typically surrounds large axons. Myelin thickness is related to the speed at which an axon can transmit electrical impulses (Rushton, 1951; Arbuthnott et al., 1980b), and for this reason myelin sheath characteristics are also of interest (Webster, 1971; Arbuthnott et al., 1980a,b; Fried and Hildebrand, 1982; Fried et al., 1982; Smith et al., 1982; Friede and Beuche, 1985; Demirer et al., 2006). Axon and myelin characteristics are commonly found using images of nerve cross-sections in which the tissue has been stained to visualize cell membranes and myelin. These visible structures are then identified and measured. This process of identifying and labeling regions of interest in an image is known as segmentation. Given that a single nerve can contain many thousands of axons and that an application can require the analysis of tens or hundreds of nerves (Schmalbruch, 1986; Vogt, 1996), it is important to develop methods to obtain the needed measurements efficiently from nerve images.

There are many methods for segmenting images of nerve cross-sections. Before the advent of computers, photographs of several representative sample areas from each nerve cross-section were printed out on large sheets of film or paper, and the axons identified and measured by hand (Webster, 1971; Hildebrand and Hahn, 1978; Boyd and Kalu, 1979; Arbuthnott et al., 1980a,b; Fraher, 1980; Fried and Hildebrand, 1982; Fried et al., 1982). By extrapolating from these sample areas to the whole nerve, the axonal characteristics of the nerve were approximated. Due to the time-consuming nature of this completely manual method, it was generally not feasible to analyze the entire nerve cross-section but only small sections.

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With the advent of more advanced computer technology, it was possible to manually identify and trace each axon in several representative sample areas using a computerized stylus or tablet on a printout or computer screen, and then program the computer to calculate the relevant measurements (Dunn et al., 1975; Bronson and Hedley-Whyte, 1977; Karnes et al., 1977; Smith et al., 1982; Friede and Beuche, 1985; Friede, 1986; Schmalbruch, 1986; Ewart et al., 1989; Hoffmeister et al., 1991). As hardware and software improvements continue to be made and new types of algorithms are developed, it has become possible to automatically identify, rather than manually trace, most or all of the axons in digital images. Algorithms using techniques such as template matching (Frykman et al., 1979), edge detection (Ellis et al., 1980; Zimmerman et al., 1980; Usson et al., 1991), active contours (Fok et al., 1996), zonal graphs (Romero et al., 2000), neural networks (Jurrus et al., 2010), and region growing (Zhao et al., 2010) have been produced to automatically identify axons and myelin in nerve images based on their shape or grey-level characteristics. These methods are faster than manual techniques, allowing more sample areas to be analyzed – for example, the measurement of 1000 axons can be reduced from 1 day to 1 h (Usson et al., 1991). In some cases, especially in the case of smaller nerves, these more efficient methods allow analysis of all axons in the nerve cross-section (Usson et al., 1991; Romero et al., 2000; Weyn et al., 2005). There have even been several studies using advanced segmentation techniques to identify and track axons in three-dimensional image sets (Jeong et al., 2009; Jurrus et al., 2010). Still, many approaches require specialized equipment, such proprietary analysis systems (e.g. Hunter et al., 2007). Few methods are entirely automated as some form of user input, for example manual addition or deletion of axons, is generally required to ensure accuracy. In fact, because of the inherent variability present in biological samples, manual confirmation of the results through the process of supervised segmentation is often desirable (Zimmerman et al., 1980; Auer, 1994).

Analyzing several sample areas from a nerve, rather than whole-nerve images, to obtain values representative of the whole nerve can introduce bias into the results. Because of their size, larger axons are more likely to intersect the edge of the sample area and be cut off, making the results biased towards small fibers (Larsen, 1998). As well, the size distribution of axons can vary in different parts of the nerve, with some areas having a higher percentage of large axons and other areas having a higher percentage of small axons (Saxod et al., 1985; Torch et al., 1989a). While these problems can be ameliorated by using more and larger sample areas, and appropriate sampling strategies, accuracy and effectiveness could be greatly improved by analyzing all axons within a nerve rather than extrapolating whole-nerve values from a limited number of sample areas (Saxod et al., 1985; Torch et al., 1989a).

Most work on segmentation of nerve images has focused on light microscope images (Dunn et al., 1975; Frykman et al., 1979; Ellis et al., 1980; Zimmerman et al., 1980; Usson et al., 1991; Auer, 1994; Mezin et al., 1994; Campadelli et al., 1999; Romero et al., 2000; Weyn et al., 2005; Urso-Baiarda and Grobbelaar, 2006; Hunter et al., 2007) or, less commonly, transmission electron microscope (TEM) images (Vogt, 1996; Vogt and Trenkle, 1998; Jurrus et al., 2010; Zhao et al., 2010). Light microscopy uses easily available low-cost equipment, while TEM is higher-cost and requires more specialized equipment but is capable of very high-resolution images (Bronson et al., 1978). Both these methods require samples to be cut into thin slices, which can be very difficult in large-diameter nerves. In addition, TEM imaging is only possible for samples of less than 3 mm in diameter, which would require large-diameter nerves to be divided into several smaller samples. A good alternative to light microscopy and TEM is the use of a scanning electron microscope (SEM). Unlike TEM imaging, which produces images by detecting electrons passed through the sample, SEM imaging produces images by detecting electrons bounced off the surface of the sample. This technique allows thicker sections to be imaged than either of the previous two methods and can therefore be used to effectively image larger-diameter nerves in which thin sections would be difficult to prepare without specialized equipment. It is also capable of imaging sample areas of several centimeters in diameter, eliminating the need to divide large-diameter nerve samples. While there have been a number of segmentation and analysis methods developed for light microscope and TEM images, to our knowledge, there has been no robust method developed to analyze whole-nerve images acquired using an SEM.

Our goal was to develop an efficient method for quantifying axon and myelin size distributions in an image of an entire nerve cross-section. We chose SEM imaging to enable examination of an entire nerve cross-section including those of nerves that are very large. For ease of use, we required that our algorithm be simple, intuitive, and not necessitate the use of proprietary software. To ensure accuracy and enable operator verification, we chose a supervised semi-automated method to segment axons and myelin in the SEM images, then used the resulting data to determine nerve fiber number, size, and myelination. In the remainder of this paper, we first present details of our method's six main steps: image acquisition, image stitching, axon segmentation, myelin segmentation, quality control, and data output. We then demonstrate the method's performance in analyzing a single rat fascicle and discuss its strengths and limitations.

2. Methods

2.1. Image acquisition

Following standard methods of sample preparation, we acquired sciatic nerve samples from a rat perfused with fixative containing 4% paraformaldehyde and 1% glutaraldehyde then further preserved the samples in the same fixative before staining them with osmium tetroxide and embedding them in plastic resin (More et al., 2010). A Bausch & Lomb 2100 Nanolab SEM imaged the embedded nerves at 1665× magnification to obtain 512 × 477 pixel images. The microscope scanned at 10 kV using a spot size of 7, the 'low 6' resolution setting, and the backscatter detector. Prior to nerve imaging, we confirmed the accuracy of the microscope scalebar with a standard test sample of known size. Nerves had total diameters of approximately 1.75 mm, with fascicle diameters ranging from approximately 0.1 mm to approximately 1 mm. Due to instrument limitations, we could not acquire one single image of the entire nerve cross-section but instead scanned through the cross-section to obtain a set of overlapping sub-images with identical size and resolution. The amount of overlap on each side of each sub-image was approximately 12–20% of the sub-image size to allow sufficient overlap to align adjacent images and allow cropping.

2.2. Image stitching

SEM imaging produced a set of sub-images for each nerve cross-section which fit together in an overlapping grid-like fashion, with the approximate position of each sub-image known. The edges of the sub-images were cropped before further processing to remove presence of edge distortion. To combine the cropped sub-images into one image showing the entire nerve cross-section, we developed an algorithm which used normalized cross-correlation to align the sub-images and stitch them together into a single image (Fig. 1). While other studies have reported more sophisticated image stitching methods (Tasdizen et al., 2010; Vogt and Trenkle, 1998), we found that cross-correlation was simple, fast,

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