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Binary imaging analysis for comprehensive quantitative histomorphometry of peripheral nerve

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Abstract

Quantitative histomorphometry is the current gold standard for objective measurement of nerve architecture and its components. Many methods still in use rely heavily upon manual techniques that are prohibitively time consuming, predisposing to operator fatigue, sampling error, and overall limited reproducibility. More recently, investigators have attempted to combine the speed of automated morphometry with the accuracy of manual and semi-automated methods. Systematic refinements in binary imaging analysis techniques combined with an algorithmic approach allow for more exhaustive characterization of nerve parameters in the surgically relevant injury paradigms of regeneration following crush, transection, and nerve gap injuries. The binary imaging method introduced here uses multiple bitplanes to achieve reproducible, high throughput quantitative assessment of peripheral nerve. Number of myelinated axons, myelinated fiber diameter, myelin thickness, fiber distributions, myelinated fiber density, and neural debris can be quantitatively evaluated with stratification of raw data by nerve component. Results of this semi-automated method are validated by comparing values against those obtained with manual techniques. The use of this approach results in more rapid, accurate, and complete assessment of myelinated axons than manual techniques.

Keywords: Binary imaging analysis; Peripheral nerve; Semi-automated nerve morphometry; Histomorphometry

1. Introduction

Nerve histomorphometry, the measurement of attributes on a prepared nerve section, has long provided important contributions to peripheral nerve research. The ability to quantitate nerve features allows a potentially unbiased way to evaluate nerve characteristics in cases such as regrowth or pathology, and yields data less subject to variability than more subjective measures such as functional recovery. Despite their importance and widespread use, histomorphometric techniques vary widely.

The ideal histomorphometry method is accurate, complete, efficient, easy to use, and inexpensive. Depending on a researcher's needs, these attributes are weighed differently and different programs are produced, perhaps with particular attention to cost (Urso-Baiarda and Grobbelaar, 2006) or to speed (Romero et al., 2000). Here, we present the histomorphometry method our lab implemented in 1989 and has continued to refine, to evaluate both non-injured and pathological nerve sections during our work on peripheral nerve regeneration (Fig. 1). This approach is driven by a desire to evaluate by direct measurement as many nerve attributes as possible and to address the fiber debris and non-viable fibers frequently seen in our pathological nerve sections using a highly customizable, rapid, computer-based technique.

We customized a semi-automated binary imaging analysis method to avoid the limitations seen with other techniques such as the active contour model (Fok et al., 1996) or common clustering technique (Costa et al., 1997). Often in peripheral nerve analysis, we encounter differing nerve fibers sizes, non-neural elements, and other thresholded gray-level profiles which are not well characterized and therefore can result in significant error during analysis. In the active contour model, fibers in close

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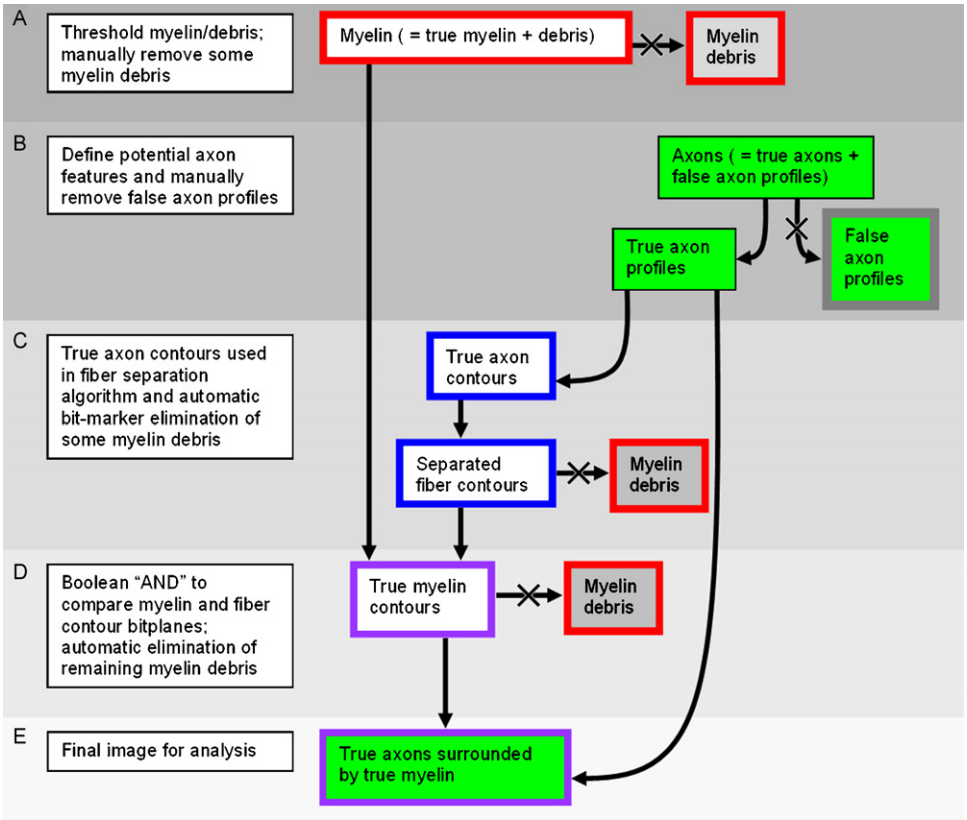

Fig. 1. Image processing algorithm. Outline and background colors of components correspond to the bitplane colors as described in the text. For a detailed description, please refer to the following subsections in the semiautomated histomorphometry section of Section 2. (A) Thresholding and manual fiber debris elimination; (B) axon definition and manual feature elimination; (C) and (D) fiber separation and further delineation of non-myelinated profiles; (E) mathematical morphometry.

proximity may not be separated and non-viable fibers are not distinguished from viable ones. As a result, there is little verification of the identity of fibers and false positives can arise. A similar critique pertains to the clustering technique. In this method, the nearest neighbor approach is used as an automatic separation mechanism. In contrast to the axon-based method of nerve fiber separation, the older cluster method does not make use of clearly discrete axons; it instead uses the whole fiber for identification. The cluster method has not been formally compared with axon-based counting in the literature, but in our experience, it is prone to undercounting of fibers and is therefore less robust. We present and validate our novel approach to nerve morphometry, a method that is fast, reliable, facile, and available to peripheral nerve laboratories. Most importantly, our method is the only published technique assessed and validated with pathologic nerve sections.

2. Materials and methods

Adult male Lewis rat (Harlan Sprague–Dawley, Indianapolis, IN) sciatic nerves were used in this study. All sections were randomly selected from prior experiments evaluating non-injury and pathologic conditions which had the approval of the animal studies committee at our institution. Selected nerve models included: an un-injured sciatic nerve, a section distal to a transection site 1 month after repair, and a section of a nerve allograft after 3 months *in situ* in an animal treated with FK506.

2.1. Tissue preparation

Harvested nerves were fixed in 3% EM grade glutaraldehyde (Polysciences Inc., Warrington, PA) at 4 °C, postfixed with 1% osmium tetroxide and serially dehydrated in ethanol. Specimens were embedded in Araldite 502 (Polysciences), and cut into 1-μm cross-sections with an ultramicrotome (LKB III Produkter A.B., Bromma, Sweden). Sections were stained with 1% toluidine blue dye, and mounted on slides for imaging. It is important to note that excellent fixation is required for accurate analysis.

2.2. Equipment

A Hitachi CCD KP-M1AN digitizing camera was mounted on a Leitz Laborlux S microscope with a manually controlled stage. A 100× oil immersion objective lens (Leitz) was used to produce a digital image at a final magnification of 1000×, with a pixel size of 0.125 μm.

The Leco IA32 Image Analysis System (Leco, St. Joseph, MI), and its earlier versions, has been used in our lab since 1989. Originally developed for analysis of compound metals, the program's measurement capabilities can be naturally extended to address nerve features. Although measurement of properties relevant to metallurgy such as grain size and porosity are built into the program, its true strength lies in the ability of the user to compose custom calculation routines (macros), expanding its analytical potential to nerve parameters.