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Comparison of Different Microscopic Sample Preparation and Imaging Techniques for Visualization of Connective Tissue Components in Peripheral Nerve

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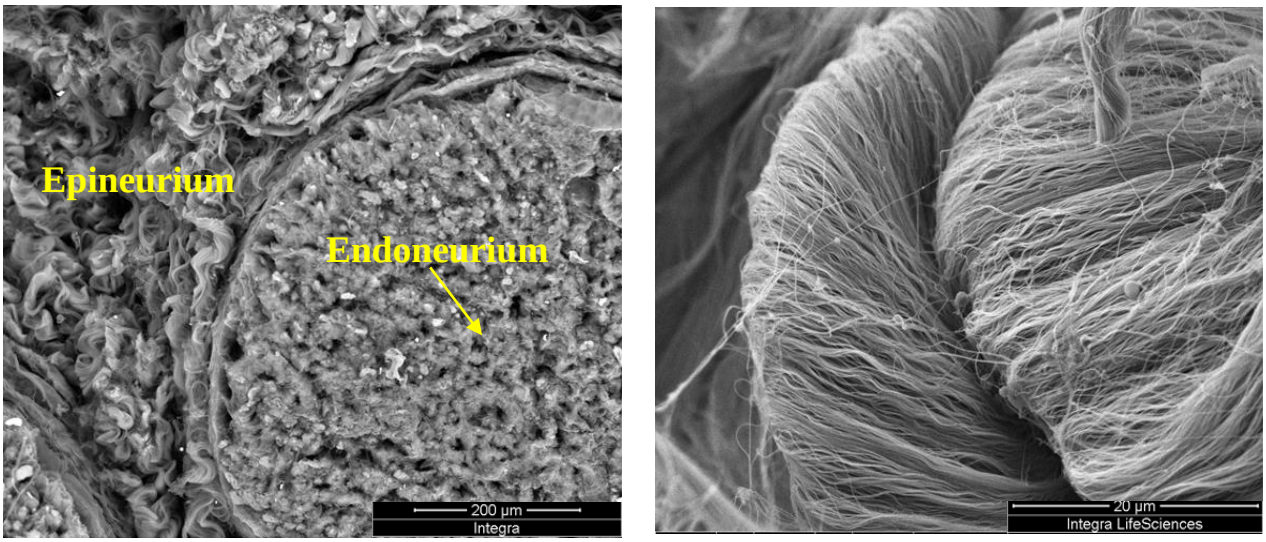
Surface structural analysis is an essential step in evaluating the morphology and structural organization of complex biological materials, such as the connective tissues of peripheral nerves. Such microscopic observations predominantly rely on Scanning Electron Microscopy (SEM) [1], Atomic Force Microscopy (AFM) [2], and micro–Computed Tomography (micro-CT) [3, 4]. Hydrated tissue samples pose a significant challenge to SEM imaging. Solvent dehydration and critical point drying are used extensively to prepare biologic samples for SEM imaging [5, 6], with the intent to produce dry artifact-free specimens preserving the fine details of the specimen structure. Recently, two methods have been developed that allow specimens to be prepared for SEM analysis that are structurally close or equal to their native hydrated state thereby promising potential artefact free observations, these methods are: replacing the water via ionic liquids (ILs), also called an ambient-temperature molten salt (ASTM) which possesses low vapor pressure that can be withheld in vacuum [7] and the NanoSuit® method that coats the specimen to block liquid escape while under vacuum [8]. In this paper, we examine which method will provide the most suitable recipe for imaging beam-sensitive, wet biological materials and provide comparison with micro-CT as a complementary technique to SEM to evaluate the quality of the sample preparation technique.

Two sample preparation techniques, critical point drying (CPD) and ionic liquid exchange (1-Ethyl-3-methyl-imidazolium tetrafluoroborate at 50% concentration), were compared to prepare fresh nerve samples for imaging using conventional SEM in Hi-Vacuum mode. For CPD-SEM experiments, accelerating voltage, spot size, working distance, dwell time, and detectors were optimized for optimal image resolution and signal-to-noise ratio at high power operation. Low magnification images were collected on the FEI Quanta 250 SEM with MAPS (Modular Automated Processing System) software using a solid-state low kV, high contrast back-scattered electron detector (vCD) at a working distance 10mm, a voltage of 20kV, spot size 6, and dwell time 30µs. High magnification images were collected using the Everhart Thornley secondary electron detector (ETD) at a working distance 3-6mm, a voltage of 5kV, spot size 3, and dwell time 60µs. A series of magnifications were taken to visualize the fiber bundles' surface structure, presented in **Figure 1**. For IL-SEM experiments, due to the low vapor pressure and relatively high ionic conductivity offered by IL which acts as a thin conductive layer on sample surface, no sputter coating was necessary as opposed to the traditional CPD prepared samples [7]. IL-SEM images were collected on the Zeiss Auriga-40 FIB-SEM using a SE2 detector at working distance 10mm, a voltage of 5kV, and 250 pA beam current. High-resolution images comparing CPD-SEM, IL-SEM, and complementary data from micro-CT were included in **Figure 2**. Our data showed that the high-resolution images provided by CPD-SEM reveal many interesting morphological details including the ultrastructure of individual collagen fibrils and collagen fiber bundles forming the

connective tissue components of the epineurium, perineurium, and endoneurium [9]. To our knowledge, such high-resolution SEM imaging of collagen fibrils and fiber bundles within connective tissue of peripheral nerves have not been previously reported.

We demonstrate that CPD-SEM technique provides high-quality images with preservation of fine topographical details. Our initial data showed promising results using IL-SEM, however, further method development is necessary to better preserve the fine topographical details. Moreover, despite the high-resolution imaging allowed by CPD, one of its drawbacks is the extensive processing time the technique requires: up to 48 hours fixation and the succeeding washing and acetone gradient exchange. Both CPD and ILs sample preparation processes are destructive in nature as samples need to be cut into small sections. The desire for non-destructive and three-dimensional imaging technique to complement SEM observation have led to including the use of micro-CT in our analysis of these materials. Specifically, we demonstrate a 3D reconstruction and segmentation method for visualizing tissue organization within the entire volume of a nerve segment (~4mm diameter x 4mm height). The advantage of micro-CT 3D reconstruction is the ability to non-destructively study the three-dimensional internal structure of intact nerve fasciculi with high voxel resolution. Sample preparation is minimal and data collection is relatively fast. Coupled with advanced computational analysis, micro-CT 3D reconstruction can elucidate the internal structural characteristics of peripheral nerve connective tissues.



A) **B)**

Figure 1. High magnification CPD-SEM images of nerve fascicles and connective tissue components revealing ultrastructure of individual collagen fibrils and fiber bundles within the epineurium and endoneurium at A.)200x and B.) 3000x. 2-3mm thick nerve samples were cut using razor blades when slightly frozen, fixed for 48 hours in 1% Glutaraldehyde at 4°C, acetone series exchange, then dried using CPD, and sputter-coated for Hi-Vac SEM imaging.

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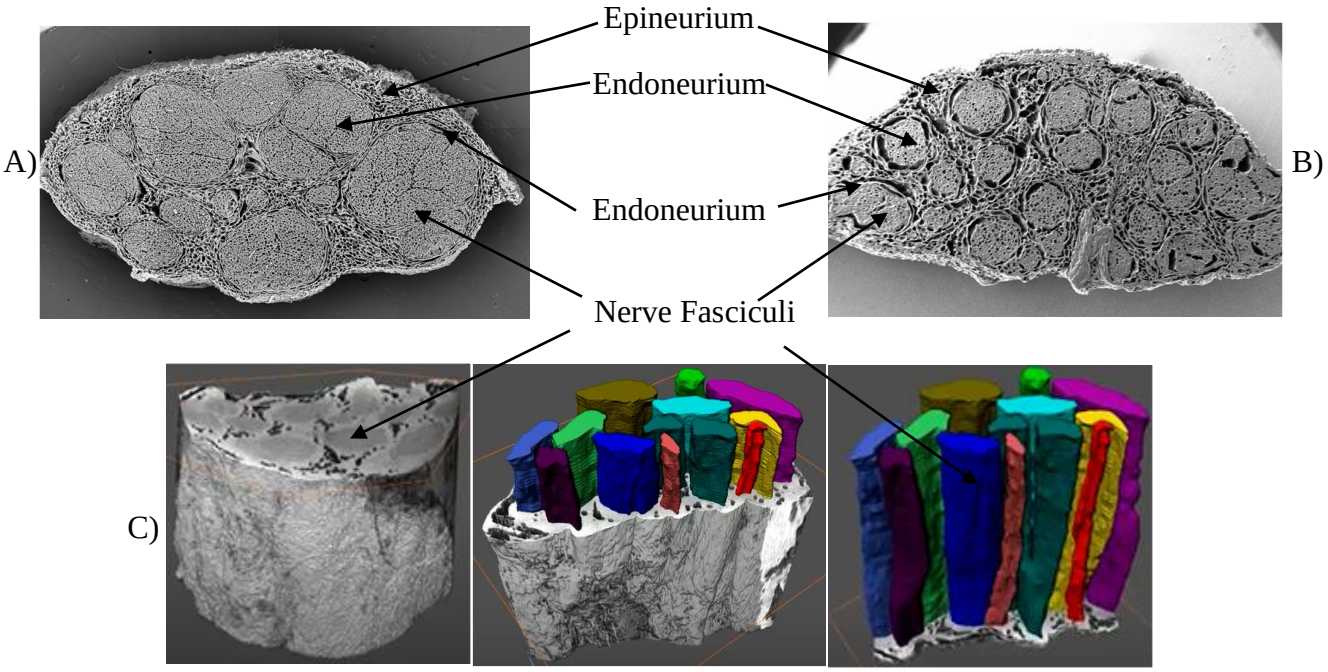

Figure 2. Comparison of different SEM sample preparation techniques for the visualization of nerves. A.) A CPD-SEM image and B.) An IL-SEM image of a sliced nerve specimen (~4mm diameter x ~1-2mm thickness/height) revealing detailed surface structure of individual nerve fascicles and connective tissue components; C.) A micro-CT 3D reconstruction and segmentation of a whole segment of nerve (~ 4mm diameter x 4mm height) revealing internal structure of intact nerve fasciculi.

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