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High-resolution fluorescence microscopy of myelin without exogenous probes

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ABSTRACT

Myelin is a critical element of the central and peripheral nervous systems of all higher vertebrates. Any disturbance in the integrity of the myelin sheath interferes with the axon's ability to conduct action potentials. Thus, the study of myelin structure and biochemistry is critically important. Accurate and even staining of myelin is often difficult because of its lipid-rich nature and multiple tight membrane wraps, hindering penetration of immunoprobes. Here we show a method of visualizing myelin that is fast, inexpensive and reliable using the cross-linking fixative glutaraldehyde that produces strong, broad-spectrum auto-fluorescence in fixed tissue. Traditionally, effort is generally aimed at eliminating this auto-fluorescence. However, we show that this intrinsic signal, which is very photostable and particularly strong in glutaraldehyde-fixed myelin, can be exploited to visualize this structure to produce very detailed images of myelin morphology. We imaged fixed rodent tissues from the central and peripheral nervous systems using spectral confocal microscopy to acquire high-resolution 3-dimensional images spanning the visual range of wavelengths (400–750 nm). Mathematical post-processing allows accurate and unequivocal separation of broadband auto-fluorescence from exogenous fluorescent probes such as DAPI and fluorescently-tagged secondary antibodies. We additionally show the feasibility of immunohistochemistry with antigen retrieval, which allows co-localization of proteins of interest together with detailed myelin morphology. The lysolecithin model of de- and remyelination is shown as an example of a practical application of this technique, which can be routinely applied when high-resolution microscopy of central or peripheral myelinated tracts is required.

Introduction

Myelin is a critical element of the central and peripheral nervous systems of all higher vertebrates. As nervous systems evolved to be more complex, high densities of conducting axons required a design shift in favor of much smaller energy-efficient fibers, with higher conduction velocities. The solution was myelination, which is a tight wrapping of axons by multiple layers of lipid-rich membrane synthesized by Schwann cells in the PNS and oligodendrocytes in the CNS. The myelin wraps are interrupted at regular intervals by nodes of Ranvier, where the unmyelinated axolemma, rich in Na channels, is exposed to the extracellular space; it is here that action currents are regenerated allowing rapid electrotonic current flow to the next node, repeating the excitation/conduction cycle, and effecting saltatory action potential propagation (Debanne et al., 2011; Nave and Trapp, 2008).

Thus, an intimate coordinated relationship must exist between axon and myelinating glia during development and maturity, for the normal process of early myelination to succeed, and for mature myelin to be maintained in adulthood. By inference, any disturbance in the integrity of the myelin sheath has deleterious consequences for the ability of an axon to conduct action potentials, with a reduction in velocity initially, increasing the risk of conduction failure as a result of acute demyelination (Park et al., 2011). Numerous peripheral and central disorders exhibit demyelination of axons as either a primary pathology, or display a component of myelin disruption as part of their pathophysiological complex. Prominent examples include multiple sclerosis in the CNS (Compston and Coles, 2008; Waxman, 2006), a variety of demyelinating peripheral neuropathies such as hereditary Charcot–Marie–Tooth disease (Murakami et al., 1996) or acquired Guillain–Barre syndrome (Dematteis, 1996), leukodystrophies (Poser, 1978), and a host of prevalent disorders where injury to myelinated axons forms an important component of the pathophysiology (e.g. stroke (Schmidt et al., 2004), spinal cord injury (Norenberg et al., 2004), brain trauma (Su et al., 2012), Alzheimer's disease (Gold et al., 2012) and even neuropsychiatric disorders such as schizophrenia (Davis et al., 2003; Karoutzou et al., 2008)). The study of myelin structure and biochemistry is therefore critically important for understanding normal development of the nervous system and the many disorders that adversely affect it.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; MAG, myelin associated glycoprotein; NF, neurofilament; PLP, proteolipid protein; PBS, phosphate buffered saline; PFA, paraformaldehyde; RGB, red green blue; SHG, second harmonic generation; YFP, yellow fluorescent protein.

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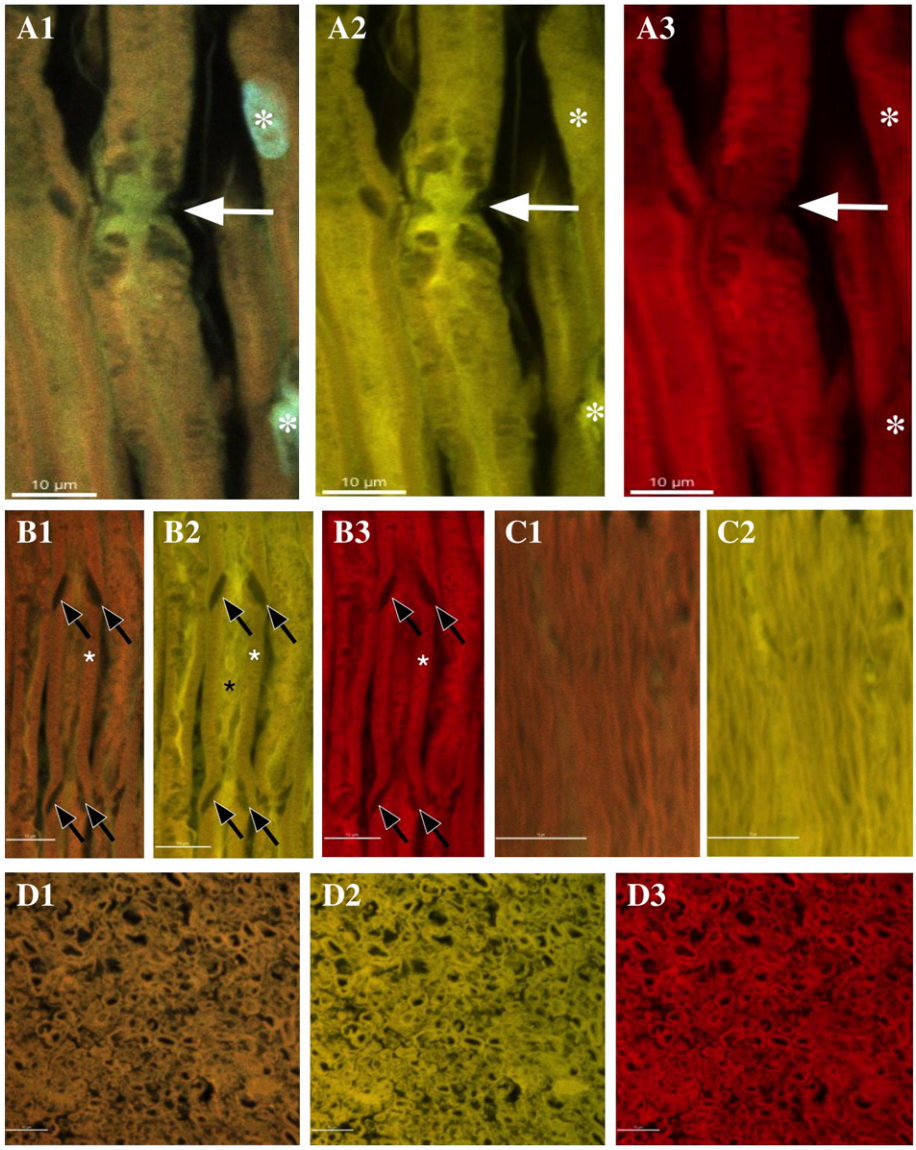

Fig. 1. Glutaraldehyde fixation induces broadband fluorescence that is excitable over a wide range of wavelengths. The induced fluorescence allows for detailed visualization of CNS and PNS tissues. A1–3: Truecolor images of teased nerve fibers of rat ventral root excited by 403, 488, and 637 nm laser lines, respectively. Anatomical structures are visualized, such as a node of Ranvier (white arrow) and Schwann cell nuclei (stained with DAPI, white asterisk). B1–3: Truecolor images of longitudinal section of rat dorsal roots excited at 403, 488, and 637 nm, respectively. Black arrows indicate Schmidt–Lanterman incisures, white asterisks indicate the myelin sheath, and black asterisks indicate the axon. C1–2: Truecolor images of rat optic nerve excited by 403 and 488 nm laser excitation. D1–3: Truecolor images of rat dorsal column excited by 403, 488 and 637 nm laser lines, respectively. Scale bars 10 μm.

Historically, a variety of methods have been developed to visualize the myelin sheaths of axons within the CNS and PNS by reactions with its phospholipids or lipoproteins (Xiang et al., 2005). Examples of colorimetric histological myelin stains include Weigert–Pal's iron hematoxylin method (Donkelaar et al., 2011), Kluber and Barrera's luxol fast blue staining (Kluver and Barrera, 1953) and staining with osmium tetroxide (Di Scipio et al., 2008). Additionally, Pereyra and Roots (1988) showed that tetracycline could be used as a stain for myelin in formalin-fixed nerve fibers, possibly via a mechanism involving myelinic calcium. All of the methods above are conventional light microscopy techniques that involve a variety of reagents and vary greatly in time requirements, ranging from several hours to weeks. More recently, immunohistochemistry using antibodies against a variety of myelin proteins (e.g. myelin basic protein (MBP), myelin proteolipid protein (PLP)) has gained favor, as well as non-immune fluorescent lipophilic fluorescent probes such as FluoroMyelin™ (Kanaan et al., 2006), various lipophilic dyes such as squarylium near-infrared dyes (Xiang et al., 2005), Nile red (Vejux et al., 2007) and Nile blue (Teo et al., 2011). More advanced techniques such as coherent anti-Stokes Raman scattering microscopy (Imitola et al., 2011) yield high quality microscopic images of myelin, but are technically demanding and not amenable for routine use. Here we describe a simple technique based on intrinsic fluorescence that develops in response to glutaraldehyde fixation of myelinated nerve fibers (Reynolds et al., 1994). This method has not gained wide acceptance because the emission is very broadband, and therefore interferes with other labels that may be used for co-staining. However, using spectral microscopy, we show how such bright myelin fluorescence can be easily separated from other emitters, yielding an effective method for labeling myelin that is compatible with multiple staining with other fluorophores, without exogenous dyes or immunolabels.

Materials and methods

Animals and tissue preparation

Experiments were performed in accordance with Canadian Council on Animal Care guidelines for the care and use of animals. Adult Long–Evans male rats (200–250 g, Charles River, Montreal, Canada), mice