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Development and Utility of a Spectral-based Approach to Peripheral Nerve Myelin Analysis

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Development and Utility of a Spectral-based Approach to Peripheral Nerve Myelin Analysis

by

Joey Kevin Grochmal

A THESIS

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Abstract

The overall aim of this thesis is to fill a specific methodological gap for the study of neurobiology. Our goal is to develop and apply a spectroscopic technique that can be used to study the living myelin of the peripheral nervous system. Just as modern scientists can appreciate the composition of a distant galaxy by the emission properties of the light that we receive from it, we likewise aim to glean information about living myelin biochemistry using quantifiable light principles. We suggest that the photophysical properties of myelin-incorporated solvatochromic dyes can be exploited to probe the biochemical composition of living myelin. To this end we analyze several potential dye candidates for maximum efficacy and consistency (Chapter 3). After demonstrating the superiority of Nile Red in these regards, we use this dye to probe the biochemical environment of early remyelination, both in-vitro and in-vivo, using high-resolution spectral confocal microscopy (Chapter 6). This combined work required the adaptation of a focal demyelination injury model to be amenable to cell graft therapy (Chapter 4), and also the development of a novel method of intravital imaging of the peripheral nervous system (Chapter 5). Our results demonstrate a consistent bi-phasic evolution of myelin spectra during early regeneration, both in-vitro and in-vivo. In total, this thesis presents a novel technique for probing the chemistry of PNS myelin with light, and is applicable to scenarios unworkable by existing methods of lipid chemical analysis, namely in the study of living nervous system.

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And thank you to my father, for fueling my interest in science all those years ago.

Dedication

This work is dedicated to my wife, Kathryn. From the moment I approached her with my intentions to pursue a Doctor of Philosophy degree, she has been unwavering in her support. She has been there through all of my trials, my failures, my set backs, my frustrations, the many late nights and the busy weekends, holding our family upright and keeping my feet on the ground. I love you honey. You know me better than I know myself sometimes, and for this I am grateful. Thank you for encouraging me through my self-doubt toward the culmination of this work. This work has been hard won, and it is as much your victory as mine.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ANOVA	Analysis of Variance
ASP	Average spectral peak-by-pixel population
bFGF	Beta fibroblast growth factor
BFP	Blue fluorescent protein
BSA	Bovine serum albumin
CARS	Coherent anti-stokes Raman scattering microscopy
CMV	Cytomegalovirus
CNS	Central nervous system
CW	Continuous wavelength
Deep-OCM	Deep optical coherence microscopy
DiI	Cell tracker CM-DiI
DLPC	Dilauroylphosphatidylcholine
DMEM	Dubelco's modified eagle media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ECM	Extracellular matrix
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EMG	Electromyography
ER	Endoplasmic reticulum
ESMD	Environmentally sensitive membrane dyes
F12	Ham's F-12 Nutrient Mixture
G	Gravity
GFP	Green fluorescent protein
HEK	Human embryonic kidney
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme
ICT	Intramolecular charge transfer
KO	Knock out
LUV	Large unilamellar vesicles
LV	Lentivirus
MAG	Myelin associated glycoprotein
MALDI	Matrix associated desorption ionization
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NA	Numerical aperture
NF	Neurofilament
NGF	Nerve growth factor
NRSSA	Nile red solvatochromic shift assay
OD	Overdose
p# (eg. P3)	Post natal day # (eg. Post natal day 3)

P0	Myelin protein zero
PBS	Phosphate buffered saline
PDL	Poly-d-lysine
Pen/Strep	Penicillin and streptomycin
PI	Polarity index
PLP	Proteolipid protein
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system
RER	Rough endoplasmic reticulum
SCAP	SREBP cleavage activation protein
SD	Standard deviation
Shi	Shiverer (gene)
SKPs	Skin-derived precursor cells
SKP-SCs	Skin-derived precursor Schwann cells
SREBP	Sterol regulatory element binding protein
TESPA	3-Triethoxysilylpropylamine
VSV-G	Vesicular stomatitis virus glycoprotein
YFP	Yellow fluorescent protein

Epigraph

Science is spectral analysis. Art is light synthesis.

Karl Kraus

Chapter One: **Introduction and Experimental Aims**

1.1 General Introduction

Myelin, produced by the glia cells of the central (CNS) and peripheral (PNS) nervous system, is a multi-layered membranous biological structure of great physiological importance. Myelin ensheathes axons, which allows for the rapid and efficient propagation of action potentials; when damaged, it can result in debilitating neurological disease, as is evidenced by multiple sclerosis and other demyelinating conditions^{16,145}. The production of new myelin is a key step in efficient nervous system regeneration³⁶, with implication for recovery from trauma¹⁰, demyelinating conditions^{12,179}, and almost any other injury to the PNS and CNS. In these conditions, study of myelin is classically performed by limited histological and morphometric analysis of dead, fixed tissue.

However, as interest in the study of living cells and tissue expands, fluorescence-based techniques for the analysis of tissue biochemistry have become increasingly important^{33,97}. For example, groundwork experiments for this thesis (now published) have demonstrated that Schwann cell-like Skin-derived precursor cells (SKP-SCs) may induce improved morphologic parameters of myelin recovery, when used as a Schwann cell graft in a focal demyelination model⁶⁰. It is also apparent that gold-standard non-invasive testing (electrophysiology) may not be sensitive enough to capture this benefit (figure 4b). When we eventually consider how we shall measure the success of stem cell grafts in human injury models, and considering the current limitations of non-invasive testing, a minimally invasive and low morbidity procedure for the

determination of myelination efficacy may be a key step toward determining the success of these grafts. This introductory chapter will provide the necessary background for placing our efforts to develop such a technology in the context of nerve injury, stem cell therapy, myelin biochemistry, and the current advances in intra-vital imaging techniques.

1.2 Peripheral Nerve Injury and diseases of the PNS myelin

Direct PNS trauma is a common injury, and has an overall prevalence of approximately 2.8% in multi-trauma populations¹¹⁶. Most reports have determined that the affected population is primarily young (<55 years old), hence contributing to an overall large societal burden^{116,150}. In the US alone, peripheral nerve injury contributes to 8 648 000 restricted-activity days, and 4 916 000 bed/disability days annually¹⁶¹. Although the treatment of these injuries has advanced much in the past 100 years³⁸, outcomes for higher grade injuries remain poor⁹⁴.

In simple terms, nerve injury of Sunderland grade 3 and above involves the damage of neural connective tissue elements, to the extreme of complete nerve transection (injury Grade 5) (Figure 1-1). These higher injury grades often fail to display spontaneous regeneration, due to either a gap in the nerve, or the formation of scar tissue and neuroma¹⁰². In these instances, after resection of the damaged segment, and when the nerve ends are not amenable to primary closure, alternative techniques must be used to bridge the nerve gap. These have included autologous nerve graft (gold standard), as well as the use of bioengineered tissue constructs (for a review, see Evans, 2001)³⁸.

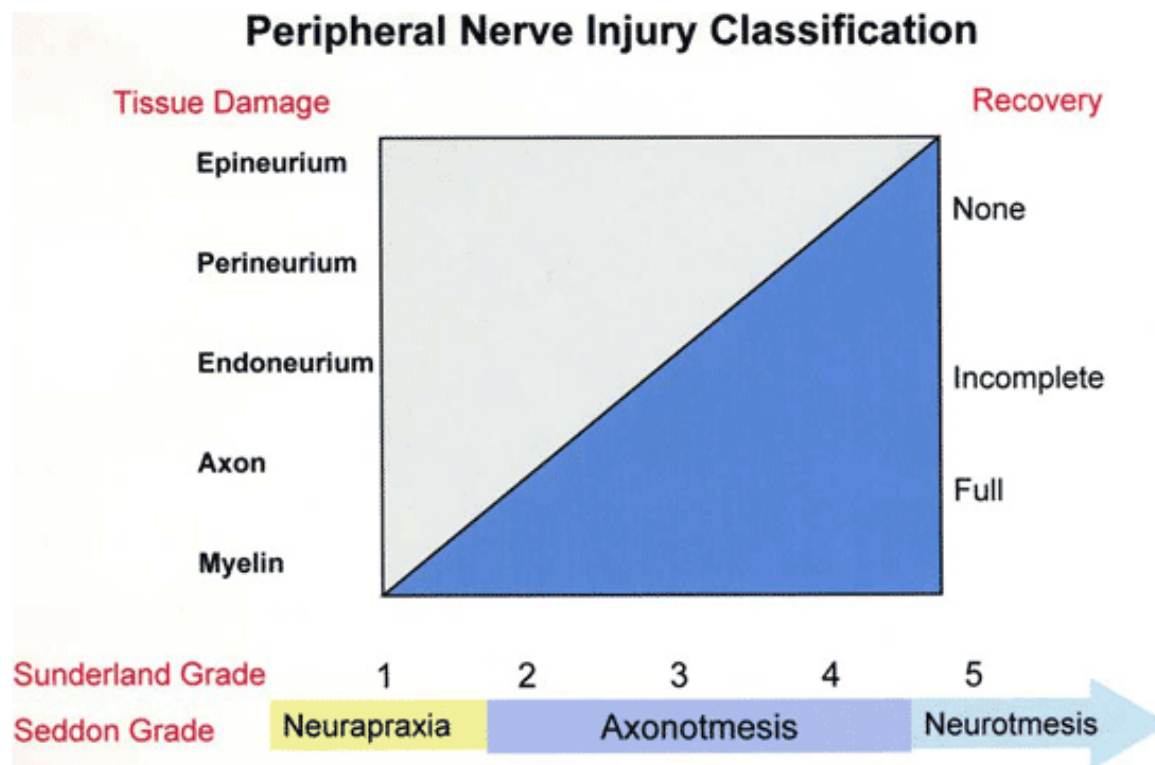


Figure 1-1 Peripheral Nerve injury grading (adapted from Burnet and Zager²⁴). Low injury grades involve either focal demyelination (Grade 1, Neurapraxia), or axonal damage (Grade 2, Axonotmesis), with preservation of the nerve microarchitecture, including the basal lamina scaffolding. Higher grades of injury indicate progressive damage to these structures, including endoneurium disruption (Grade 3), perineurium disruption (Grade 4), as well as a complete nerve transection injury (Grade 5). Higher grades of injury are associated with progressively poor clinical outcomes.

1.2.1 Schwann Cells Support the Regenerative Environment

There are a multitude of factors that affect the ability of a nerve to regenerate in the context of nerve injury^{28,45}. Assuredly, intrinsic responses of the axons themselves are essential for the conversion from a signalling to a regenerative neuronal phenotype.

Up regulation of growth related genes occurs post-injury, with neurotrophic factors playing key roles in axonal growth and survival²⁸. On the other hand, the maintenance of a supportive regenerative environment is also fundamental for successful axonal regeneration⁴⁶. To this end, the role of the Schwann cell has been extensively studied.

Following injury to the PNS, Schwann cells support the regenerative environment in a multitude of ways. Schwann cells quickly change their phenotype from a myelinating to a growth supportive mode^{45,106}. They also proliferate post-injury in the distal stump, and migrate to form the bands of Bungner²³, Schwann cell lined columns of basal lamina tubes. This proliferation peaks at about 3 days post-injury for previously myelinating Schwann cells^{29,45}. These dedifferentiated Schwann cells that have obtained the growth supportive phenotype secrete neurotrophins and cytokines¹⁵¹ while producing ECM proteins that are growth supportive²². They can also function in a macrophage type role, and play a major role in phagocytosis of debris in the early time points after PNS injury¹²⁸. However, studies have demonstrated that the capacity for endogenous Schwann cells to maintain a growth supportive phenotype decreases over time¹⁸⁰ which may be a major reason for the decreased capacity of chronically denervated nerves to support axonal regeneration¹⁶².

1.2.2 Schwann Cells as Therapy

Although effort has been made to improve regeneration by re-invigorating and re-transplanting chronically denervated Schwann cells^{146,147}, the clinical impracticality of this approach has encouraged scientific focus towards the supplementation of injury models with cultured

Schwann cells. With the discovery of Schwann cell mitogens such as heuregulin, as well as the advancements of cell-culture techniques, isolated Schwann cells have become amenable to expansion and subculture⁹⁵. These cultured Schwann cells maintain a Schwann cell phenotype through repeated sub-culture passages, and also maintain the ability to myelinate neurites in vitro^{95,111}.

Schwann cell graft treatment of both PNS and CNS injury has been studied extensively. As examples in the peripheral nervous system, Guenard et. al⁶² demonstrated the superiority of Schwann cells in supporting axonal regeneration through a guidance channel repair of the rat sciatic nerve. This finding is mirrored in regeneration across basal lamina grafts seeded with Schwann cells⁶⁵. Acellular tendon and muscle grafts demonstrated improved rates of axonal regeneration when seeded with Schwann cells, as compared to graft alone in a sciatic nerve model¹¹⁴

On a complimentary note, transplanted Schwann cells have demonstrated the ability to remyelinate demyelinated lesions of the central nervous system^{13,84}; they have also been analyzed in more severe central nervous system injury models. Xu et al.¹⁷³⁻¹⁷⁵ demonstrated improved regeneration into Schwann cell-seeded guidance channels in thoracic cord injury. Schwann cell transplant also contributes to improved axon survival and myelination in spinal cord contusion models¹²². In addition, human culture-expanded Schwann cells have demonstrated potential for improving axonal regeneration in rat spinal cord transection injury models⁶³.

The inability to rapidly expand donor isograft Schwann cells for transplantation into an injured host is currently a limiting factor for the clinical translation of Schwann cell transplant therapy¹¹⁴; in turn, allograft Schwann cell transplantation results (outside of immunosuppression) have remained sub-optimal. Guenard et. al⁶² demonstrated the superiority of syngenic vs. heterologous allograft, which is consistent with other observations¹⁰⁵ that immune compatibility of donor Schwann cells is an important determinant of graft efficacy (Rodriguez et al, 2000). In addition, the invasive nature of Schwann cell biopsy (which requires a nerve injury), combined with the less than desirable potential for donor neurologic morbidity, currently makes these cells less than ideal for clinical therapeutics.

1.2.3 SKP cells as Schwann cell replacement therapy for injury of the nervous system

Considering the aforementioned limitations of Schwann cell therapy in translational work, research has focused on finding an alternative Schwann-like cell type for transplantation. The ideal cell type would be easily harvested with minimal or no morbidity, be rapidly expandable in culture, and able to survive and act in the capacity of a dedifferentiated Schwann cell in the injured nervous system. To date, many different types of cells have been investigated to fit this role^{1,26,77,82,113}. The detailed literature regarding the beneficial effects of these and other cell types in peripheral nerve regeneration has been reviewed extensively elsewhere^{61,108,161}

Of special interest to our lab, Skin Derived Precursor Cells (SKPs) are multipotent stem cells that reside in the dermal papillae of mammalian skin¹⁵², and have successfully been harvested and cultured from both rodents and humans^{152,153}. These cells can take on the morphology of Schwann cells when exposed to the proper differentiation media in combination with

mitogens¹⁵³ and in this state are referred to as SKP-SCs. These cells can myelinate axons in culture¹⁰⁴ and appear to myelinate both PNS axons in normal and Shiverer mutants distal to crush injury¹⁰⁴. When transplanted into an acellular nerve grafts, these cells secrete neurotrophins, and appear to improve early nerve regeneration (as compared to media injection)¹⁵⁹. These cells may also be capable of re-invigorating the regenerative environment of chronically denervated nerve¹⁶². Coupled with their ease of harvest and culture expansion¹⁶⁰ these cells of interest demonstrate great promise as a therapeutic option for the treatment of nerve injury and demyelination disorders.

A strong focus of this thesis is the solvatochromic analysis of myelination by both Schwann cells and SKP-SCs. However, the applications for the non-invasive study of stem cell myelination (or myelination in general) are perhaps far broader than the study of peripheral nerve injury. Multiple sclerosis is a result of inflammation-associated destruction of the myelin sheath, and is usually a progressive condition for which there is no known cure. Stem cell therapy for the remyelination of CNS lesions^{115,170} has been one potential avenue of therapeutic investigation for this condition⁹⁶. Spinal cord injury is another major disease entity whereby remyelination by stem cells is a current area of research²¹ Furthermore, inherited specific diseases of myelin proteins may also derive benefit from stem cell remyelination therapy⁹³. While we have decided to investigate the spectral phenomena inherent in PNS remyelination, the principles and techniques may have far spread applicability to the study of regenerative neurobiology.

1.3 Biology of PNS Myelin

1.3.1 Structure

PNS myelin is a fascinating biological structure composed primarily of compacted lamellae of a specialized bi-layer membrane, and is formed by Schwann cells². Ensheathment of axons by this structure allows for fast axonal conduction to take place, and is also important for the maintenance of axonal integrity¹³⁹. Adjacent Schwann cells participate in the formation of the Nodes of Ranvier, which are the structural segments of the myelin-axon complex that make possible saltatory conduction (Figure 1-2⁵⁰). The myelin is present in both compact (internodal) and non-compact regions (present at paranodal regions and Schmidt-Lanterman incisures).

The biochemistry of myelin is unique in its high protein to lipid composition ratio⁵⁰. Lipids account for 70-80% mass of the dry weight of isolated PNS myelin and while there are no specific differences in the classes of lipid represented in PNS myelin, some differences exist as to the quantities⁵⁰. For example, PNS myelin is particularly rich in sphingolipids¹¹⁷. Myelin is also highly enriched in cholesterol, accounting for 20-30% of total myelin lipid¹⁷⁸. Many proteins are specific to PNS myelin, and these will be detailed below.

Myelin lamella form by the fusion of cytoplasmic and extracellular leaflets, forming the major dense and intraperiod lines respectively; compact peripheral myelin has a periodicity of approximately 12nm, or 119 angstroms from intraperiod to intraperiod line (Figure 1-3).

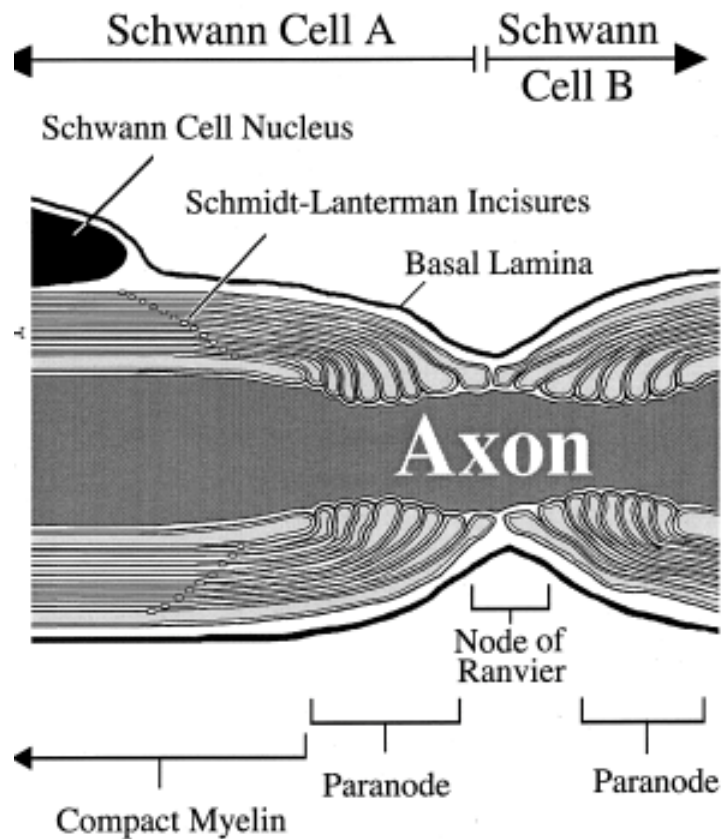


Figure 1-2: Schematic of a myelinated axon (adapted from Garbay et al.⁵⁰). Compact myelin is present in the internodal region. Nodes of Ranvier (bare axonal regions specified for ionic conduction) are formed by the apposition of paranodal regions of neighbouring Schwann cells. Paranodal myelin is non-compact, as are the Schmidt-Lanterman incisures, which likely function as transport channels between the internal and external mesaxon⁵⁰.

1.3.2 Development

Myelination of the rodent PNS is a process that begins at or near birth; at p1, the majority of rat sciatic nerve axons are associated with Schwann cells that form no myelin lamellae, though a minority do in fact form lamellae of less than 10 turns⁴⁴. Developing Schwann cells can be initially classified in to four types, depending on their stage of maturity.

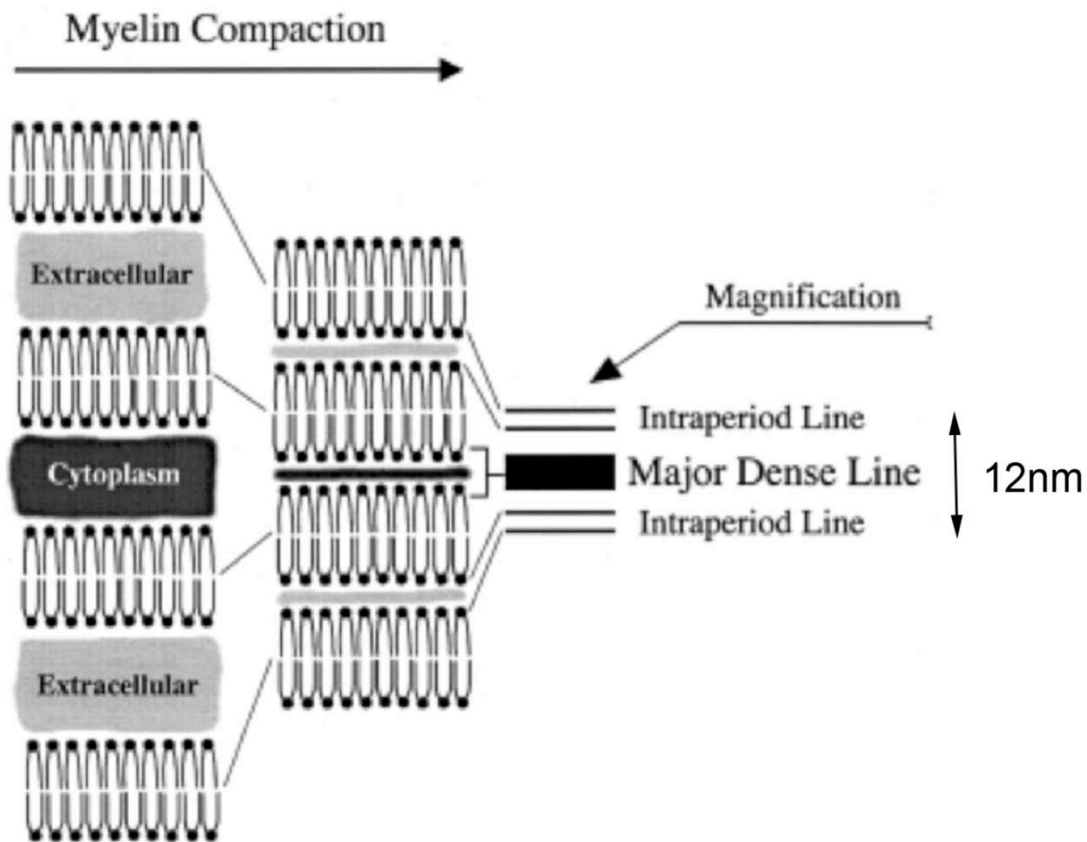


Figure 1-3: Myelin compaction involves the close apposition of extracellular and intracellular-facing Schwann cell membranes. These form the intraperiod and major dense lines respectively. PNS myelin periodicity is approximately 12nm. Adapted from Garbay et al.⁵⁰

“Fetal type” Schwann cells initially form an association with bundles of small axons⁷⁴. They then undergo mitosis to form a 1:1 relationship with axons, in a process termed “radial sorting”⁷⁵.

At this stage individual Schwann cells acquire a basal lamina¹⁶⁵ before proceeding to myelinate their respective axons, and at this stage are referred to as “pro-myelinating” Schwann cells⁴⁴.

Compaction and growth of the myelin sheath then occurs, at which point the Schwann cells are called “myelin forming”, then proceeding towards the end phenotype of “mature myelinated” over the course of several weeks⁴⁴.

Studies on post-natal development of rat sciatic nerves have delivered similar reports on the rate of PNS myelination. One account documents an exponential growth of lamellar length over the first 15-20 days, with a gradual decrease in rate of myelin formation over the next 60 days⁴⁴.

Another report documents a relatively rapid, yet constant rate of growth for myelin lamellae in the same early period, which then also levels off¹⁶⁵. These observations are consistent with other reports¹³⁷. The rate of lamella formation is approximated at 5 hours per turn for p0-p10 rats, 9 hours per turn for p10-p20 rats, and slower thereafter⁴⁴. Not all axons destined for myelination begin the process at the same time however, and in the first two weeks of development there is a large proportion of pro-myelin figures still present. This number decreases as development continues, as pro-myelinating Schwann cells mature into myelin forming Schwann cells⁴⁴

During this critical developmental period, production of compact myelin is paralleled by an up regulation in the production of the major protein components of PNS myelin, namely Myelin Protein Zero (P0), Myelin Basic Protein (MBP), Peripheral Myelin Protein 22 (PMP 22), and Myelin Associated Glycoprotein (MAG)¹⁴³. The rates and dynamics of myelin protein mRNA expression during development of the rat sciatic nerve has been previously been elucidated (Figure 1-4)¹⁴³.

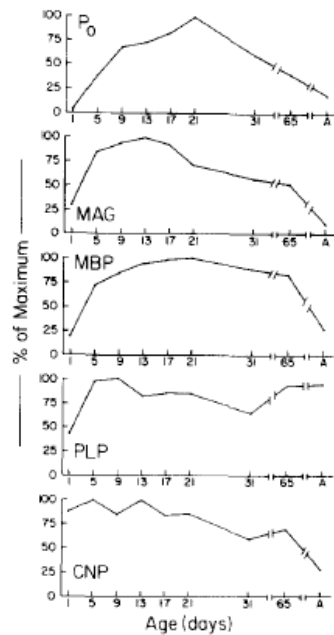


Figure 1-4: Key myelin mRNA expression during early development. Protein components of peripheral nerve myelin follow somewhat independent curves for maximal mRNA expression during early development. In particular, MAG, MBP, and PLP mRNA are highly expressed early in development (Day 5), while P0 protein reaches maximal expression after approximately three weeks. Adapted from Stahl et al.¹⁴³.

Changes also occur to the lipid content of myelin during this same developmental period. In general, the phospholipid population of young myelin contains greater proportions of shorter hydrocarbon chains (C16:0). Adult myelin fatty acids are often longer, with oleic acid (C18:1(n-9))⁵⁰ being the most common. We also know that cholesterol accumulates in a gradual fashion over the first 8 weeks of early development¹⁷⁸.

1.3.3 Myelin Proteins P0 and MBP

Recent literature has elucidated the role of P0 as a tetramer forming trans-membrane protein of the antibody lineage, that mediates the adhesion of the extracellular, intraperiod line component of compact myelin^{31,49,141,142}. P0 is synthesized in the RER, and trafficked to the forming sheath via the Golgi apparatus^{154,172}. Homozygous P0 knockout mice display a markedly aberrant myelin ultrastructure that lacks compaction as well as intraperiod lines^{53,100,142}. Indeed, hypomyelination also results from the over expression of P0 in genetically engineered mice, revealing the potential importance of potentially stoichiometric expression levels for this myelin membrane protein¹⁷². MBP usually constitutes 5-15% of total peripheral myelin protein, second only to P0¹⁴³. It is a highly cationic protein⁹⁰ that localizes to the cytoplasmic side of the myelin sheath, and in contrast to P0, is locally synthesized there via free ribosomes¹⁷².

1.4 Myelin Mutant and Knockout Mice

1.4.1 Shiverer (MBP-/MBP-) mutant mouse

In terms of MBP knockout mice, the Shiverer mouse phenotype (shi/shi) is remarkable for generalized tremors that begin at approximately p12, with other features of the condition being seizures and a shortened life span¹²⁷. These mice, while having a central nervous system that is markedly deficient in myelination, maintain the gross morphology of a normal PNS¹³¹. This is in the absence of the production of MBP¹⁵.

From a lipid biochemical standpoint, the lipid composition of Shiverer mutant mice has been previously elucidated and compared to the respective wildtypes⁴⁷. As compared to controls,

shi/shi mice have decreased levels of non-hydroxy cerebroside (↓15%) and sphingomyelin (↓15%), and increased levels of hydroxy cerebroside and sulfatide (↑15-25%). Also, Shiverer myelin contains decreased levels of cholesterol (↓10%)⁴⁷.

1.4.2 SCAP knockout mouse

The SCAP mouse is a conditional knockout of the sterol regulatory element-binding protein (SREBP) cleavage activation protein, specifically targeted to PNS Schwann cells by crossing mice bearing a floxed-SCAP gene with mice producing P0 promoted cre-recombinase starting at E14¹⁵⁶. The lack of SCAP prevents SREBP from activating genes responsible for internal cellular synthesis of cholesterol and other lipid moieties, and results in profound PNS hypomyelination, at least until early adulthood whereby they demonstrate some recovery of myelination by a hitherto unknown lipid/cholesterol source. The adult SCAP myelin is far from normal however, and had profound biochemical alterations¹⁵⁶ (phosphatidylserene, sulfatide ↓85%, cholesterol ↓85%).

Coincident with this, adult SCAP knockout myelin demonstrates an altered single point x-ray diffraction pattern consistent with a decrease in lateral lipid packing (Figure 1-5).

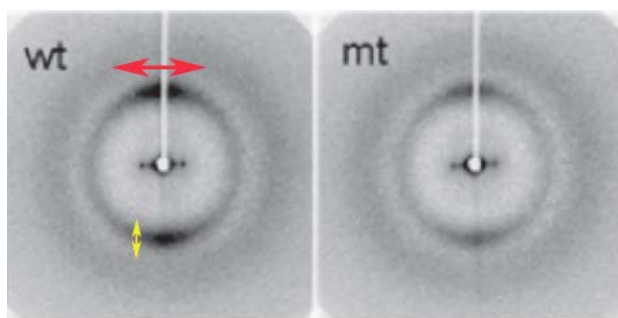


Figure 1-5 X-ray diffraction analysis of adult (P210) WT vs. SCAP myelin. Increased circumferential diffraction spacing (red arrow) and thickness (yellow arrow) are indicative of myelin increased lipid order in the wildtype myelin (adapted from Verhelihen et. al.¹⁵⁶)

1.5 Environmentally Sensitive and Solvatochromic Membrane Dyes

A rapidly expanding field biophysical science is the analysis of the composition of membranes by environmentally sensitive probes³³. These probes are fluorescent molecules that change their fluorescent characteristics (absorption/emission maxima, fluorescence intensity/duration) depending on their molecular micro-environment³³. Changes in the state of the membrane (liquid vs. liquid ordered vs. gel), as well as changes in membrane composition/polarity, are environmentally specific events that can translate into fluorescence dynamics; the specific changes observed also depend on the type of dye employed.

To understand the mechanism of action of these dyes, a basic understanding of the electrochemical forces acting on biological membranes is essential. There are three competing electrostatic forces that comprise the net environmental dipole that will be experienced by a probe³³. These are:

1.5.1 The surface potential (ψ_s)

This is the electric field experienced by a probe, due to the interactions of its chromophore with the interface formed by the membrane surface (phospholipid head groups) and the surroundings. This primarily reflects the interaction of these phospholipids with surrounding water and ions, and is dependent on membrane surface hydration and pH. Again, this potential is best measured by fluorophores whose chromophore component is at or near the surface of the membrane. An example of this style of probe is U-6, which is a modified 7-hydroxycoumarin analogue⁸⁶.

1.5.2 The dipole potential (ψ_D)

Both phospholipid molecules and water molecules have a specific ordered dipole. A dipole is the vector charge (magnitude and direction) that is inherent to a stable yet electrically polarized molecule. Due to the contributions of both surface water and phospholipids, the dipole potential localizes between the surface of the membrane and the hydrophilic core³³. The net effect of the surrounding dipoles is experienced by the fluorophore molecule (at any point in time) in the fashion of a “solvent sphere”⁹⁷ (Figure 1-6)

Certain classes of environmentally sensitive fluorophores obtain very large dipoles when excited by the appropriate wavelength of light, as compared to their base, non-excited state³³. When this occurs, the surrounding solvent sphere molecules may respond with the phenomena of “solvent relaxation”, aligning their dipoles to stabilize the new dipole of the fluorophore. The degree of solvent relaxation determines the final energy of the excited emission state of the fluorophore, as the relevant solvent relaxation occurs faster than emission (Loving et al., 2009). The greater the stability of the excited state generated by solvent relaxation, the lower the energy

(longer wavelength) of the emitted photon. This phenomenon is termed *solvatochromatism* (Figure 1-6).

Dyes that exhibit a large excitation dipole are mainly of the ICT class, which stands for Intramolecular Charge Transfer. Upon excitation, these dyes obtain a tautomeric conformation that demonstrates a much larger dipole than the non-excited state (Figure 1-7).

Common dyes of this class include NBD, Coumarin, Prodan, Laurdan, Nile Red, and Nile Blue^{33,97}, with environmental changes being reflected primarily in changes in emission wavelength maxima of these dyes.

Due in part to the large contribution of water dipoles to the solvent sphere, solvent relaxation may primarily describe water dynamics and membrane hydration, especially in regards to probes that localize themselves to the outer surface of the membrane³³. However, ICT probes have also been seen to respond to fluctuations in membrane pressure^{58,91}, curvature⁵⁸, and composition⁸⁷.

1.5.3 The transmembrane potential (ψ_T)

This potential largely reflects transmembrane differences in ion concentrations³³. The transmembrane potential is also measured by ICT probes that are usually long, rod shaped, and penetrate deep into the bi-layer. Dyes whose spectra respond to changes in the transmembrane potential are termed “electrochromic”.

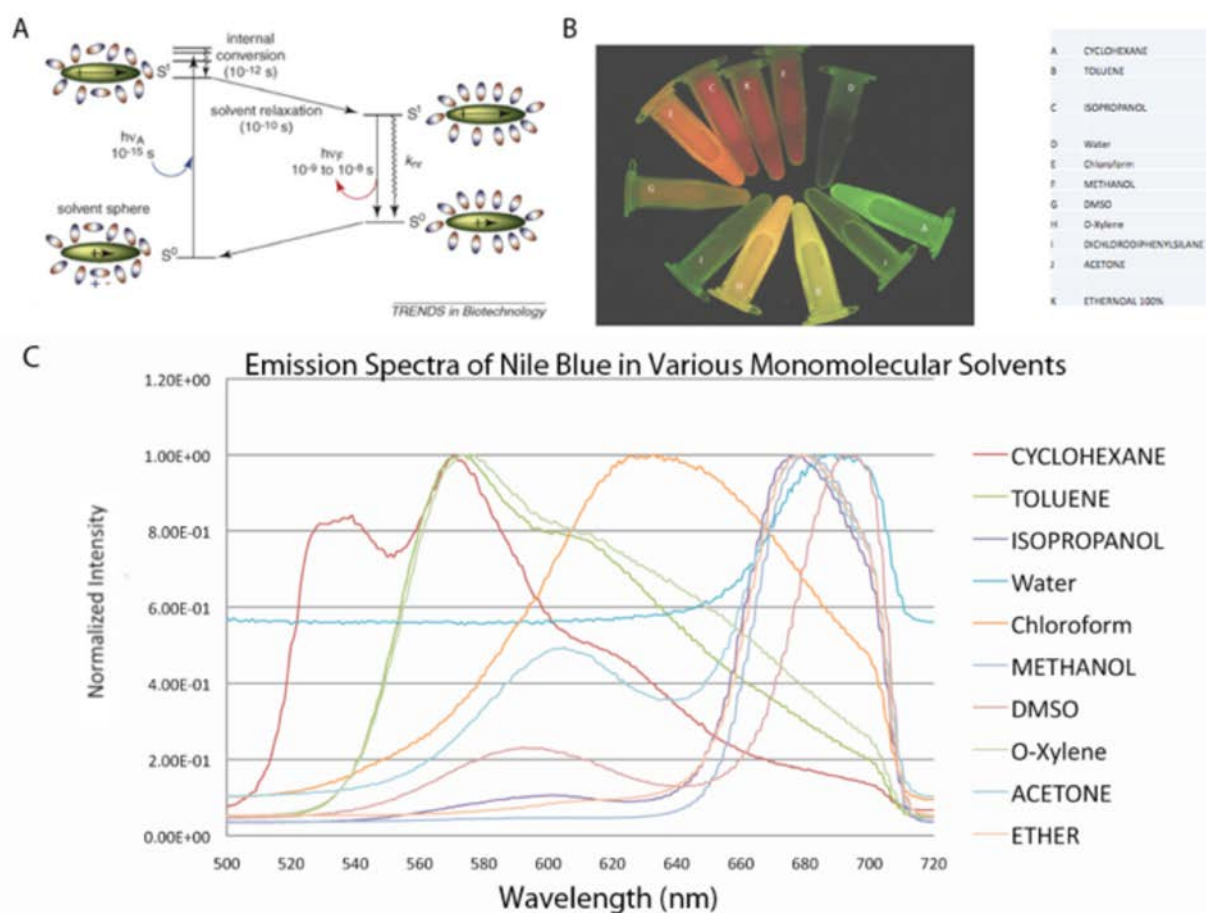


Figure 1-6 The principle of solvatochromism. A) Upon light excitation, solvatochromic fluorophores attain a large intramolecular dipole⁹⁷. The reaction of the surrounding “solvent sphere” to the dipole is a near-instant, stability-inducing interaction that decreases the energy of the excited state, proportional to the polarity of the solvent sphere. Emission wavelengths therefore vary in accordance with the polarity of the environment i.e. greater or lesser lipophilicity/hydrophobicity. B) Nile blue dye emission, as dissolved in various monomolecular solvents, under UV light excitation (Teo and Stys, unpublished, with permission). C) Nile blue emission spectra, as measured when dissolved in the solvents from panel B (Teo and Stys, unpublished, with permission).

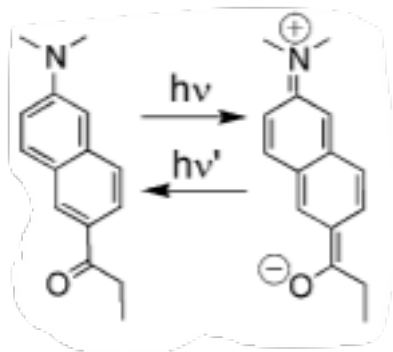


Figure 1-7 Tautomeric isoforms of Prodan. Prodan undergoes intramolecular charge transfer upon absorption of light energy to achieve a large dipole. Adapted from Dechemko et al.³³

Since the transmembrane potential has an effect on both the non-excited as well as the excited states of these fluorophores, and since the excited state may be variably influenced by the phenomena of solvent relaxation, changes in the absorption maxima of these dyes are most commonly reported when analyzing the transmembrane potential.

1.6 Nile Red

The majority of this dissertation aims specifically to examine the spectral properties of Nile Red in myelin of various compositions and regenerative states. Nile Red is classified as a benzophenoxazone, and amongst the most highly fluorescent when considering dyes of this class¹⁴⁴.

The solvatochromic character of Nile Red has been extensively investigated in the literature. In a landmark study in the Journal of Lipid Research, Greenspan and Fowler detail the fluorescent characteristics of Nile Red in multiple lipid systems, including phospholipid vesicle and lipid microemulsions⁵⁹. Within these systems, there is evidence that the addition of relatively lipophilic moieties results in blue shifted emission spectra⁵⁹. These results hold true even when looking at more complex systems, such as cellular membranes. For example, the membranes of adipocytes, rich in neutral lipids, are more blue shifted than those of hepatic microsomal membranes which consist almost exclusively of phospholipid components⁵⁹.

Nile Red analogues have also been developed for the study of lipid order and cholesterol content of experimental vesicle membranes and live cells. The engineered Nile Red dye (NR12s) has localization exclusively to the outer membrane surface, had little cellular internalization, and also demonstrated spectral shifts very similar to Nile Red in a variety of monomolecular solvents. Studies with this dye also revealed blue shifted spectra with proportional cholesterol loading, red shifted spectral with cholesterol depletion (via methyl- β -cyclodextrin), and distinctive blue shifted emission for a sphingomyelin/cholesterol lipid ordered phase vs. the lipid disordered phase (dioleylphosphatidylcholine vesicles)¹¹⁹

1.7 Spectral Confocal Microscopy

In simple terms, a spectral confocal microscope is one capable of quantifying light emission across a typically wide band of wavelengths. A standard three-channel photocube equipped confocal microscope has hard-set wavelength ranges over which all emission in said range becomes attributed to that particular “channel”. This design leads to well known problems

regarding bleed through of fluorescent signals into adjacent channels, as well as autofluorescence being attributed to specific channels, resulting in effective interpretation only with widely spaced emission of a few amount of fluorophores, and always in the presence of rigorous positive and negative control experiments. Spectral confocal microscopes were largely home-made units at the outset, designed by laboratories requiring experiment-specific enhanced functionality from their confocal units. In particular, these spectral microscopes allowed the detection and separation of emission peaks 20nm or less⁹² (Figure 1-9), which is particularly useful for detection of overlapping fluorescent protein signals. They can do this because of their ability to acquire quantitative wavelength data from up to 32 channels (vs. the standard 3-4), enabling a full and accurate spectral representation of the light data being received from the sample (Figure 1-8).

Modern spectral confocal microscopes, coupled with unmixing algorithms, are able to separate fluorescent signals that are of similar emission characteristics and of disparate intensities (Figure 1-9).

A limitation of spectral imaging concerns the acquisition times involved. Older systems captured a single spectral channel per imaging pass, requiring up to 32 individual passes to form a spectral data set. Systems improved to 8 channels of processing per imaging pass, and currently the entire 32 channel range can be processed in a single pass⁹². Although this may take only a second or two, the scan speeds are drastically slower than video rate confocal or 2-photon imaging.

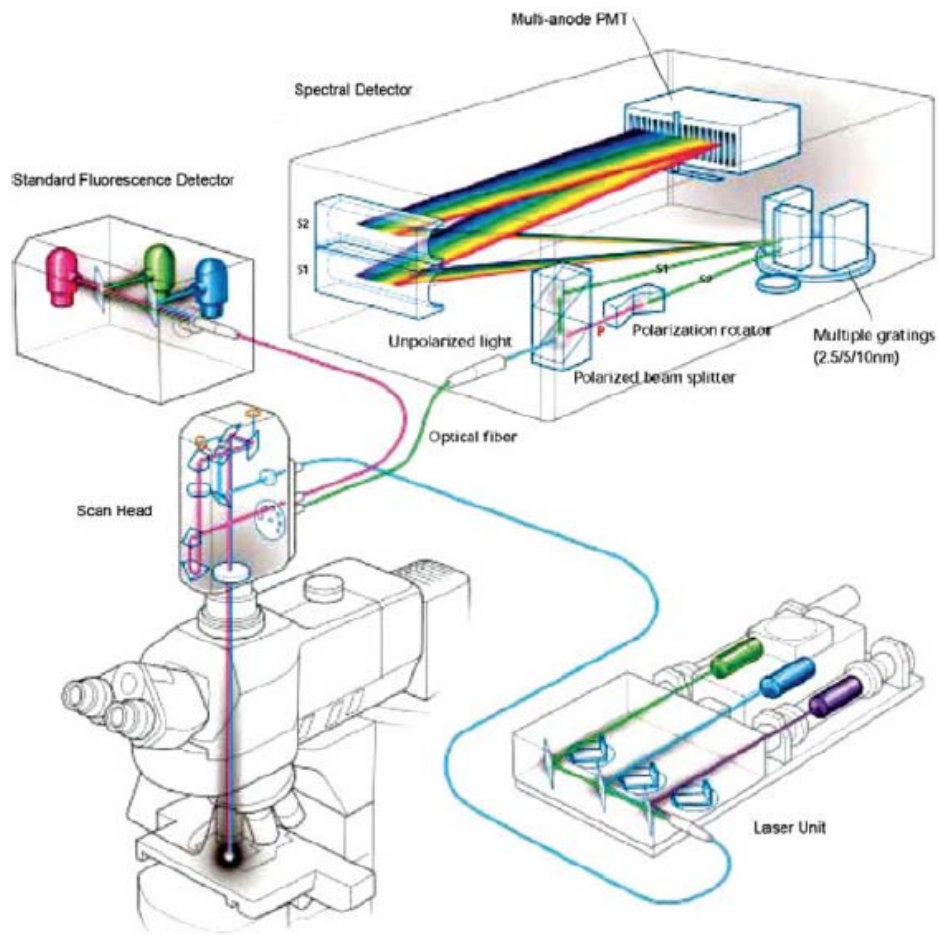


Figure 1-8 Schematic diagram of a Nikon C1si spectral confocal microscope. Light from the optical plane enters the spectrometer via fibre optic cable. Using mirrors and optics, light polarity is aligned in a single plane before being appropriately diffracted (by either 2.5, 5, or 10nm gratings) and cast upon a 32-channel photomultiplier anode array for quantification. (Adapted from Larson⁹²)

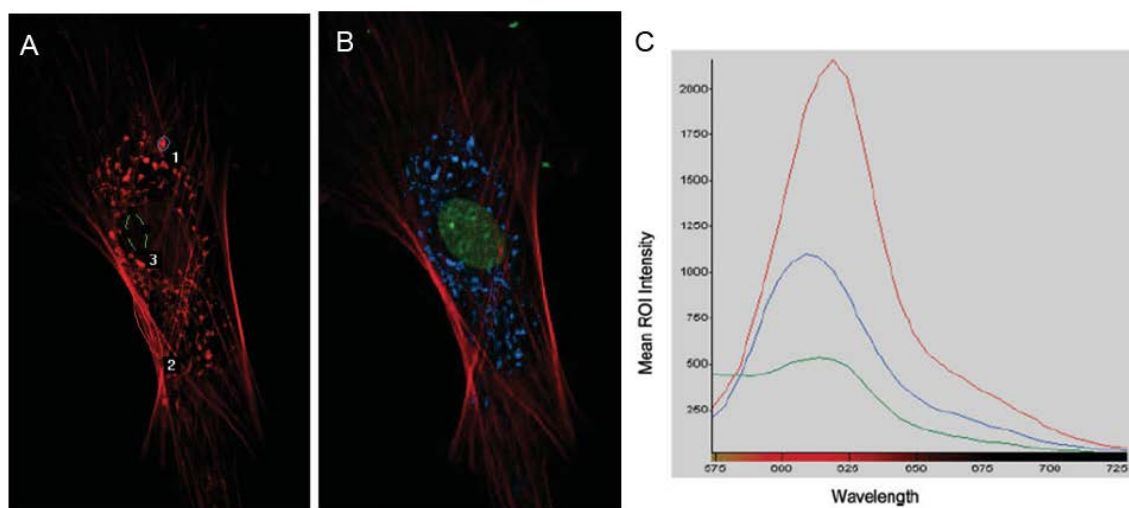


Figure 1-9 Spectral unmixing of fluorophores of similar emission characteristics.

Fibroblasts were stained with Bidopy-TRX (displayed in red), Sytox orange (Displayed in green), and Mitotracker Red (Displayed in blue). A) Truecolor confocal image b) Spectral unmix using the selection regions from A, with the component spectra displayed in their respective unmixed colors in C). Adapted from Larson⁹².

For our purposes, spectral microscopy has allowed us to capture and quantify small differences in the emission characteristics of myelin incorporated dyes, which would be otherwise difficult to follow using standard 3 channel confocal microscopy.

1.8 Goals and Hypothesis

Our goal is to establish solvatochromic membrane dyes as a tool to interrogate the composition of myelin through analysis of their spectral properties, as these dyes readily label myelin. **Our overall hypothesis is that solvatochromic dyes can serve as indicators of changes in myelin composition, as a function of maturation, injury**

and subsequent repair. Each of the 4 results chapters (below) contains specific goals and hypothesis toward this objective. We plan to investigate this hypothesis in the peripheral nervous system of the rat, towards the ultimate goal of applying this technology toward the imaging of living myelin.

Chapter 3:

Goal:

Our goal for this chapter is the elucidation of the optimal dye for study of myelination phenomena in the PNS. This dye should be reliable, consistent, and able to detect known differences in the myelin composition of developing and mutant myelin. Just as solvatochromic fluorophores are effective probes for the composition of simple membrane and vesicle systems, we predict they can also indicate changes in myelin composition.

Hypothesis:

Solvatochromic, myelin-incorporated dyes will reliably indicate the known differences of various alternate states of myelin composition.

Chapter 4:

Goal:

One goal for this chapter is to establish an injury model of focal demyelination, and to determine the ability of Schwann cell and SKP-SC grafts to myelinate axons and rescue this demyelination injury. A second goal is to identify myelin that specifically belongs to cells provided

exogenously, enabling us to define a myelin population that is undoubtedly regenerating (form an overall population that may be either regenerating or never injured).

Hypothesis:

SKP-SCs can myelinate axons in a doxorubicin induced model of focal demyelination, providing physiologic myelin that participates in mature Node of Ranvier formation.

Chapter 5:

Goal:

The goal of this chapter is to create a novel method for imaging the living peripheral nervous system of the rat, which will allow for the capture of spectral confocal images at high magnification and resolution.

Hypothesis: N/A

Chapter 6:

Goal:

The goal of this chapter is to demonstrate the utility of Nile Red spectral analysis for commenting on the regenerative maturity of myelin provided by Schwann cell and SKP-SC transplantation, particularly in regard to the imaging of living regenerative systems in-vitro and in-vivo.

Hypothesis:

Nile Red will demonstrate reliable spectral phenomena indicative of progressive myelin maturity during regeneration, and these emission characteristics can be used to follow myelination in living systems.

Chapter Two: **Common Methods**

2.1 Abstract

There are several methodologies in this thesis that are pervasive and common. In this chapter we detail these items, and provide reference for much of the common terminology.

2.2 Ethics Statement

All rats were housed independently in the University of Calgary Animal Research Center for the duration of the experiment, under normative conditions of temperature and light cycle, with free access to food and water. Greatest care was taken to minimize animal suffering and provide adequate analgesia in the post-operative period. All animal interventions were approved by the University of Calgary Animal Care Committee (ethics protocol # M08124).

2.3 Cell Culture

Established laboratory protocols were used for the harvest and primary culture of SKP-SCs^{153,159,162} (neonatal), as well as neonatal and adult Schwann cells^{85,159}. Briefly, neonatal pups (P2,3) were sacrificed by rapid decapitation, and skin sterilized with %70 ETOH. For SKPs, dorsal back skin was collected, minced in HBSS on ice, and incubated with collagenase (0.1%) for 45 minutes at 37°C. Tissue was then pulverized and rinsed with cold HBSS, and the wash then strained with a 70µm cell strainer. The filtrate (containing SKPs) was then centrifuged, and

the pellet triturated and resuspended in DMEM/F12 (3:1)(Gibco) with 1% B27(Gibco), 1% Pen/Strep (Sigma), 20ng/ml EGF (Gibco), and 40ng/ml bFGF.

To induce SKPs towards a Schwann cell phenotype, SKPs were cultured as spheres using the above media in 30ml culture flasks and passaged 3 times. They were then plated on 15cm culture dishes (BD Falcon, BD Biosciences) coated with poly-D-lysine (20µg/ml) and laminin (4µg/ml) (Both BD biosciences). The media was changed to differentiation media, consisting of Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12 3:1) with 4µM forskolin, 10ng/ml heregulin 1β, and 1% N2 supplement (Gibco, Burlington, ON, Canada).

Schwann cells were isolated from both adult and neonatal sciatic nerve for our experiments. Sciatic nerves were discarded, the epineurium discarded, and the remainder minced. These segments were placed on 35mm culture dishes in serum rich media (DMEM/F12, 10%FBS, 1% Pen Strep) to encourage fibroblast into the dish. After several days, explants were removed and placed in another 35mm culture dish, this time in low serum media (2.5% FBS). This procedure was repeated every three days until no visible fibroblast outgrowth was observed. At this point, cells were transplanted to 10cm dishes (BD Falcon) coated with laminin and PDL, and cultured in maintenance media as above.

2.3.1 BFP/GFP transduction of SKP-SCs and SCs

We used established protocols³⁰ to transduce neonatal SKP-SCs/Schwann cells with lentiviral eGFP and eBFP. We used Human embryonic kidney (HEK) 293 cells to establish the virus; these cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal

bovine serum and 1% penicillin/streptomycin, and maintained in 5% CO₂ at 37 °C. HEK293 cells were then plated onto 15 cm plates and grown to ~80% confluence.

2.3.1.1 eGFP

Using the HEPES/CaPO₄ method⁸³ we transduced the lentiviral eGFP, the viral core packaging construct pCMVdeltaR8.74 and the vesicular stomatitis virus glycoprotein envelope vector into (HEK) 293 cells. DNA containing media was replaced after 4hrs with fresh media, incubated, and collected every 24hrs x 3 harvests. Using concentrated virus we achieved transduction titres in the 1.5x10⁹ TU/mL range, as determined by epifluorescence. Cells were fax sorted to >95% expression for GFP and P75 immunolabeling.

2.3.1.2 BFP

The pTagBFP-N vector was subcloned into an existing eGFP pWPXL (addgene # 12257) lentiviral construct using 5' BamH1 and 3'EcoR1 sites. Also using HEK293, the cells were co-transfected with a mixture of pLL3.7 (addgene #11795¹³²,) containing the cloned shRNA of interest, psPAX2 and pMD2.G (addgene #12259 and #12260, Didier Trono, unpublished) using Hepes/CaPO₄ precipitation⁸³. Four hours post-transfection, the DNA-containing media was replaced with 14 mL fresh media and incubated for 24 h. The media was subsequently harvested and replaced with fresh media for a total of three harvests. Lentivirus-containing media was centrifuged at 500 xG for 5 min, 0.45µm filtered and stored at 4°C for up to 3 days. The harvested media was then pooled and ultracentrifuged at 50,000 xG for 2 hours using a SW28 rotor. The supernatant was discarded and the lentivirus pellets were resuspended in 1XPBS (15

µl per tube) and stored in aliquots at -80°C. Viral titers were tested by applying serial dilutions of lentivirus to HEK293 cells in 12-well dishes for 16 h in the presence of 8 µg/mL polybrene, followed by replacement with fresh media and incubation for an additional 24 h. Transduction efficiency was estimated by visualization of BFP fluorescence using an epifluorescent microscope, indicating viral titers of $\sim 10^9$ - 10^{10} IU/mL. Cells were FACS sorted to >95% expression for BFP and p75 immunolabeling. As BFP SKP-SCs cells were not previously characterized, we did immunolabeling to confirm the presence of BFP and Schwann cell markers (Figure 2-1).

2.4 Confocal Microscopy

Chapter specific imaging techniques are outlined in the methods section of each chapter.

2.4.1 Nikon C1Si inverted microscope

All fixed tissue analysis and in-vitro analysis was performed with this microscope, utilizing a Hamamatsu 32 Channel multi-anode photomultiplier array. The spectrometer grating was set to 10nm bin-widths for all imaging sessions, allowing the potential capture of 320nm of visible light emission.

2.4.2 Nikon A1R upright microscope

All intravital (live animal) imaging was performed with the Nikon A1R upright microscope, utilizing same spectrometer model as above, along with an Apo LWD 25x water dipping lens (NA 1.1, Working distance 2mm).

2.5 Spectral Image Processing and Polarity Index Analysis

Except when mentioned, we utilize a novel method for the quantification of spectral analysis.

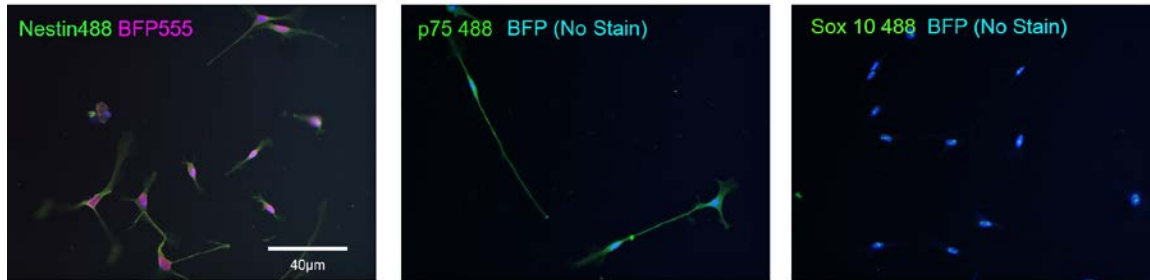


Figure 2-1 Cultured BFP transduced SKP-SCs demonstrate Schwann cell specific markers.

A) SKP-SCs stain positive for both Nestin (Alexa 488-green) and BFP (Alexa 555- magenta) B) They also are positive for p75 (green-Alexa 488; BFP–no stain) C) SKP-SCs show nuclear Sox 10 positivity as well (green-Alexa 488; BFP-no stain). The markers are expected to be positive for cells that have been differentiated toward a Schwann cell lineage. Image courtesy of Jo Anne Stratton, unpublished, with permission (Midha/Biernaskie lab).

This method is based on the determination of the peak spectral frequency for every pixel in a myelin selection region. A myelin selection region is defined by the user, and consists of a blue selection mask that encompasses one or many pieces of myelin in an image (Figure 2-2). These masks can be automatically generated, though in the case of determining the selection regions from SKP-SC and Schwann cell graft-derived myelin, all selection regions were drawn by hand after close inspection. All pixels within the blue selection mask contribute and are subject to the further analysis described below.

Each pixel essentially has its own spectrum. A pixel's spectral peak frequency is determined by Imagetruk (Image analysis software, written by Peter K Stys), whereby an analysis grid of a set size is determined, with the pixel of interest in the middle.

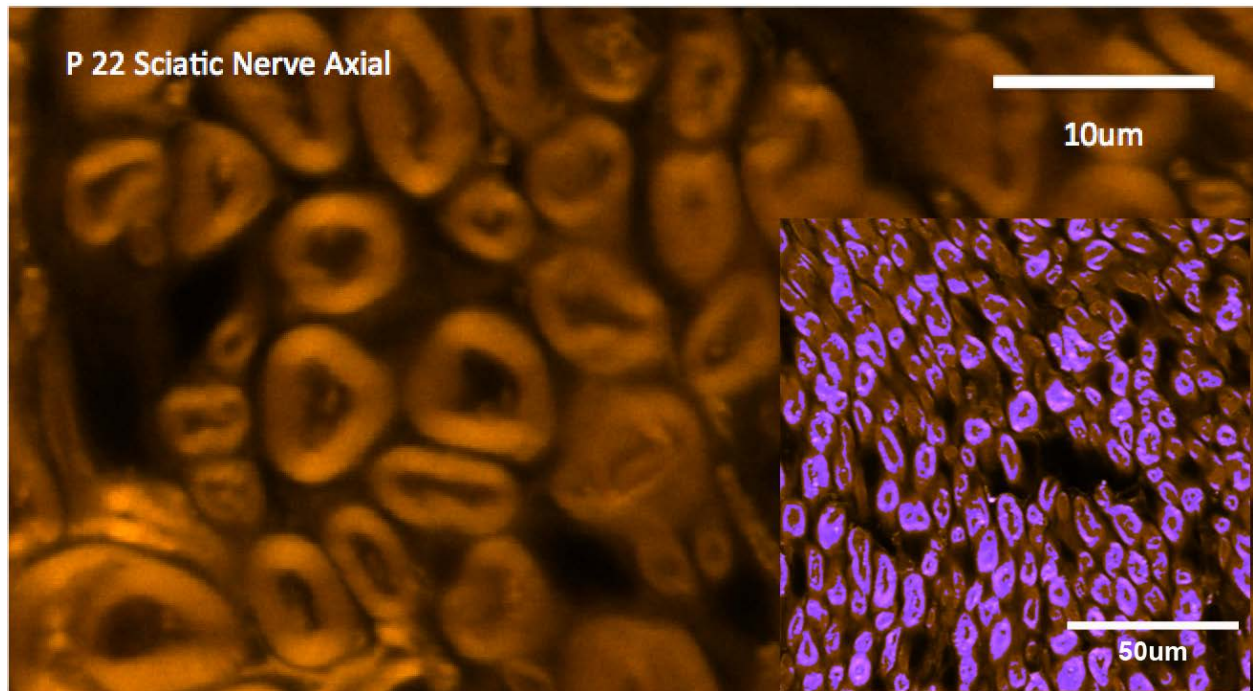


Figure 2-2 Myelin selection masks. P22 sciatic nerve, stained with Nile Red, high magnification. Inset: Lower magnification image demonstrating myelin selection regions as outlined by blue selection masks.

Using Gaussian analysis, the software proceeds to analyze the spectra of each pixel in the grid, and uses this information to extrapolate and smooth the spectral information attributed to the pixel of interest. The analysis weights contributions to be heaviest by spectra inferred closest to the pixel (and from the pixel itself), and in this manner is able to generate a pixel specific spectral curve. The peak value of this pixel-specific curve is collected for every pixel in a myelin selection region. For example, a myelin selection region with approximately 100 pixels

will generate 100 separate spectral peak data points. These data are combined into a selection region histogram (Figure 2-3).

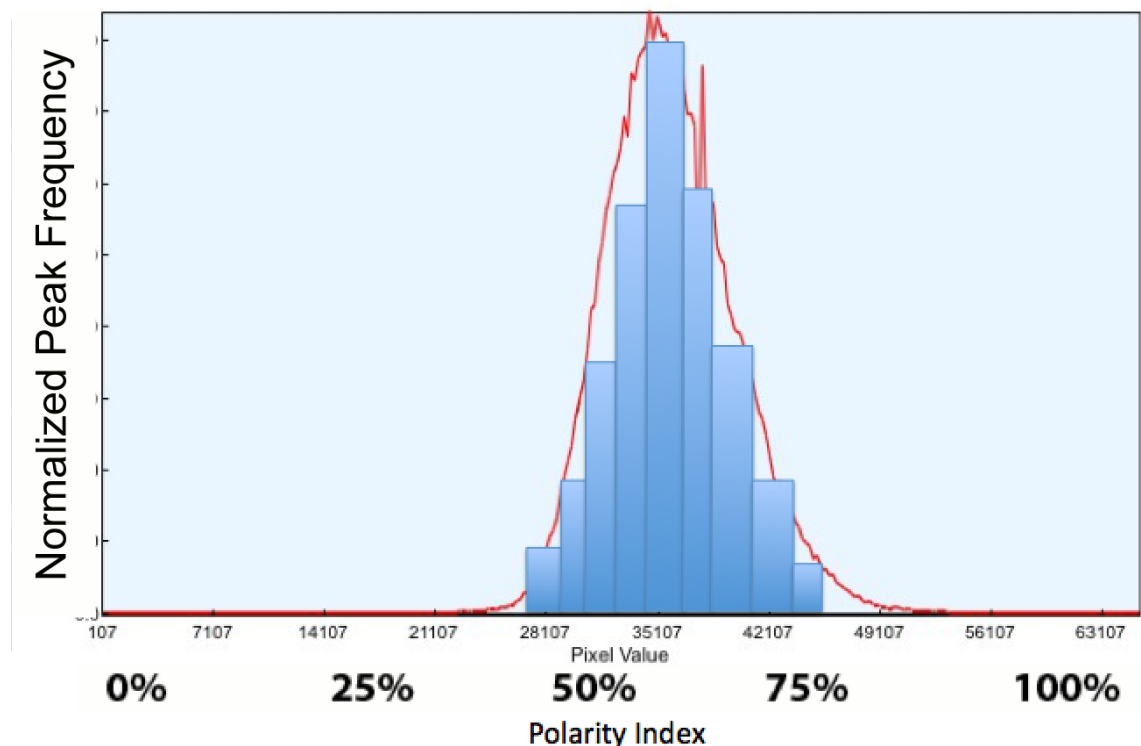


Figure 2-3 Calculation of the peak frequency curve. The pixel specific peak spectral value derives a major/minor percentage contribution from each bracket spectrum, placing it on a continuum of possible spectral peaks existing between these brackets. The pixel specific peak value contributes to a particular bin-width, forming a peak value population histogram. Visualized above is a histogram with very wide bin-widths for conceptualization. The bin widths used in the PI calculation are much smaller, representing only a fraction of a percent of the total range of possible peak spectra. With narrow enough bin-widths, accumulated histogram data takes on the appearance of a curve (red line) that is often distributed in a normal fashion. With the x-axis normalized to a percentage, and using specific bracketing spectra for the calculation, the peak of this curve is called the Polarity Index Peak value.

In addition, the word “frequency” is a somewhat misleading term for this analysis. The calculated peak value is not in reality an actual frequency, and unlike the real frequencies from which it is calculated (wavelength/nm), has no units. The value actually represents the relative position the spectral peak of that pixel would fall between two known bracketing spectra.

The investigator has the choice of which bracketing spectra to use for this computation. We decided to establish our bracketing spectra based on two opposite extremes of Nile Red spectral emission that may be observed in the study of biological nerve specimens. The Invitrogen Fluorescence Spectraviewer library was used to download the red edge (Nile Red in phospholipid) and blue edge (Nile Red in triglyceride) spectral brackets;

(<https://www.lifetechnologies.com/ca/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html>).

The derivation of spectral brackets from an external library standard also provides a means whereby other scientists may readily apply this technique to their own research. Using Imagertrak, we further moved each bracket to include a wider range of analysis, such that we could potentially analyze Nile Red emission spectra in a wide range of monomolecular solvents (Figure 2-4, 2-5). As designed by Dr. Wulin Teo (Stys lab, unpublished, with permission), this specific method computes a standardized Polarity Index for Nile Red peak spectral emission populations, and is called the Nile Red Solvatochromic Shift Assay (NRSSA) (Figure 2-5).

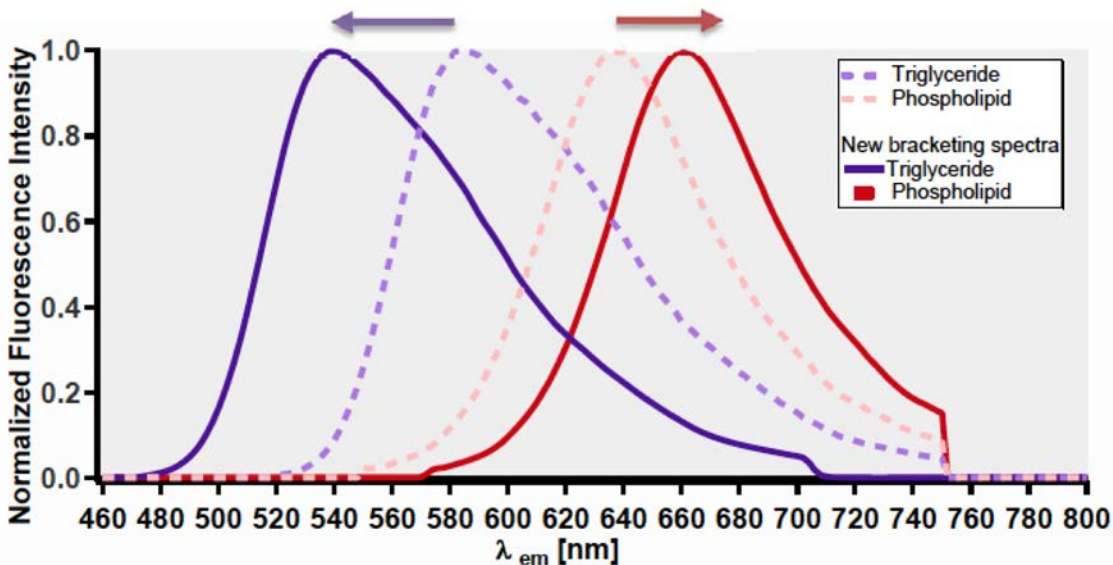


Figure 2-4 Derivation of Nile Red Polarity Index bracketing spectra. The bracketing spectra are used to define the scale of polarity index (0-100) in the NRSSA method. Triglyceride and phospholipid are selected as the bracketing spectra for all the NRSSA analysis (obtained from the Invitrogen Spectraviewer library). The range between these two spectra is extended to allow Nile Red emission in most potential solvents to resolve on the scale of polarity index. The new bracketing spectra (solid lines) cover the range between 540 nm and 660 nm. Reprinted from Teo and Stys, unpublished, with permission.

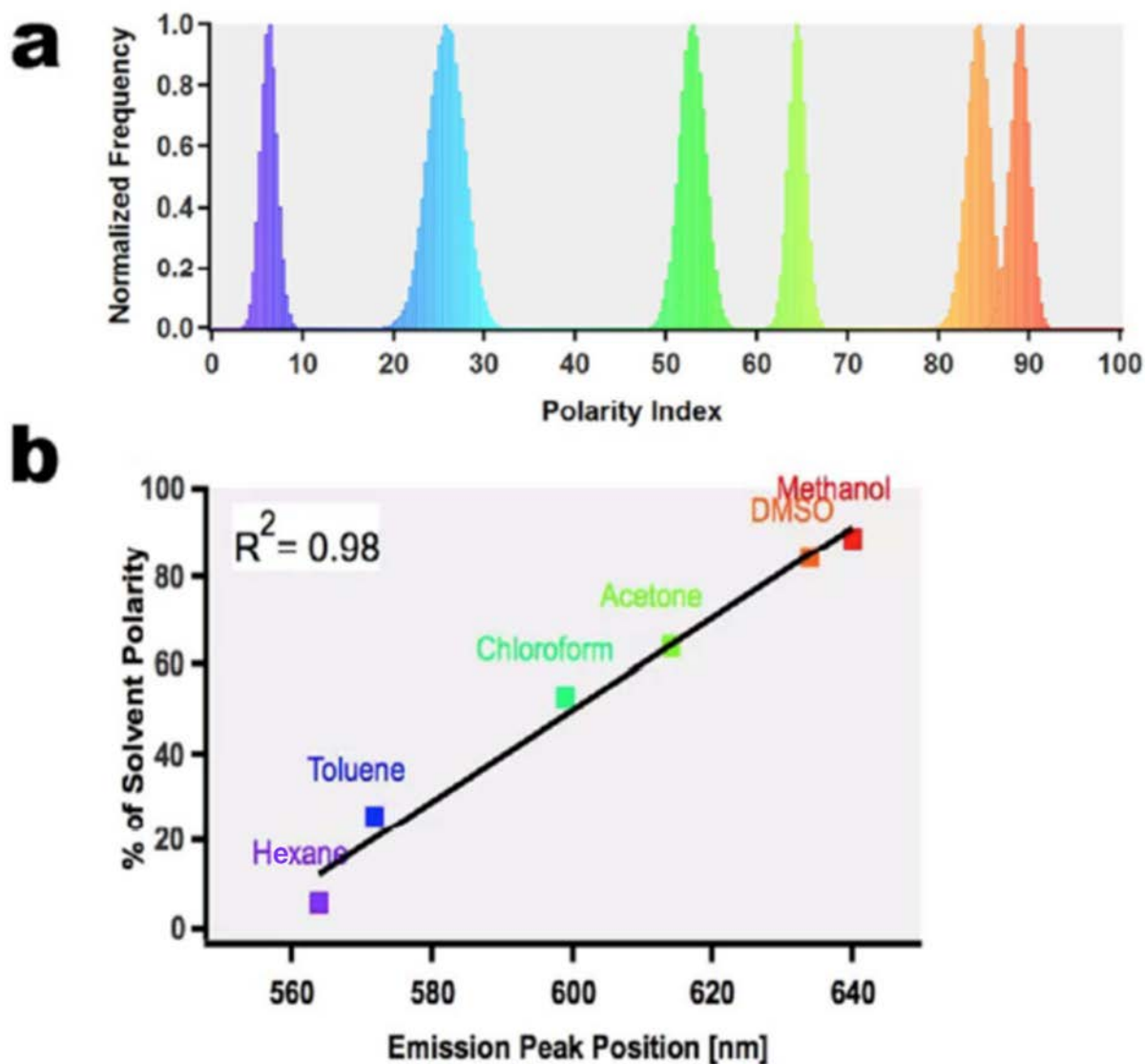


Figure 2-5 Nile Red Solvatochromic Shift Assay A) presentation of each solvent as a population in histogram by the scale of their relative polarity index (1-100) vs. normalized frequency in NRSSA analysis. B) Demonstration of the linear correlation between lambda and polarity index by plotting maximum peak of lambda and polarity index of each solvent. Reprinted from Teo and Stys, unpublished, with permission.

The concept of this style of quantified spectral analysis is pervasive to the work that follows.

When we refer to the Polarity Index value for myelin stained with Nile Red, we are referring to the spectral peak pixel values of myelin selection regions that have been analyzed and standardized using the above transformations.

Chapter Three: **Interrogation of Prodan, Laurdan, Nile Red, and Nile Blue reveals Nile Red as the optimum PNS solvatochromic myelin agent.**

3.1 Abstract

In order to qualify as an optimal solvatochromic fluorophore for the analysis of peripheral nerve myelin, the dye of interest must be reliable, consistent, easy to use, and able to elucidate known differences amongst groups of myelin that are known to be biochemically dissimilar. We tested two dyes of blue emission (Prodan, Laurdan) and two dyes of red emission (Nile Red, Nile Blue) for these particular properties over a series of experiments. Early control experiments excluded both Nile Blue and Prodan as unreliable spectral reporters for PNS myelin, alongside demonstrating that dye concentrations of greater than 10 μ M run the risk of falsely reporting on concentration-dependent spectral effects. Within these parameters, Laurdan and Nile Red were extensively tested as potential reporters of known chemical differences amongst myelin of progressive post-natal sciatic nerve time points, as well as the mutant and wildtype myelin of both Shiverer and SCAP mouse sciatic nerve. Altogether, Nile Red proved to be the more sensitive probe for reporting these differences via spectral emission properties. Coupled with the ease of use and the technical merits of Nile Red as a probe within the context of our future aims, we confirmed Nile Red to be the ideal candidate as a go-forward spectrochemical indicator in peripheral nervous system myelin.

3.2 Introduction

Many solvatochromic fluorophores readily label biological membranes, and possess a hydrophobic region allowing incorporation of the molecules into phospholipid bilayers,

including myelin. Common dyes of this class include NBD (7nitrobenz-2-oxa-1,3-diazol-4-yl), Coumarin (and analogues), Prodan, Laurdan, Nile Red, and Nile Blue ^{33,97}. By considering that myelin may act as the “solvent” for these lipophilic probes, our overall hypothesis is that myelin properties can be reported by the spectral emission phenomena of these dyes. This particular chapter’s set of experiments aims to demonstrate that myelin of disparate composition can indeed interact with these myelin-incorporated dyes, to the extent that variances in myelin composition are reflected by spectral emission shifts.

As mentioned previously, solvatochromic probes have demonstrated sensitivity to fluctuations in membrane pressure⁹¹, curvature⁵⁸, and composition⁸⁷. Most of these measurements have been in experimental membrane systems. One concern is that such eloquent dye sensitivity may be detrimental to the analysis of a multilamellar biological system, and thereby present with enough noise so as to drown useful information regarding myelin health. We designed a series of control experiments that addressed the dye-specific reliability of this emission, and also tested their respective spectral properties through varying certain key experimental parameters, such as dye concentration and manner of tissue fixation.

To elucidate dye utility, solvatochromic dyes were used to label myelin of various developmental time points. It is well known that the composition of myelin undergoes change during development^{50,143}. An effective dye should demonstrate sensitivity to these changes. In a further series of experiments in this Aim, solvatochromic dyes were used to label myelin of mutant mice. As the mutant myelin varies from control myelin in either protein content, myelin

structure,^{53,131,142,172} or lipid ordering⁴³, we expected to observe the contributions of these factors to emission signal changes.

3.3 Methods

3.3.1 Tissue processing

All sciatic nerve tissue was obtained from live animals with the exception of SCAP tissue, which was received frozen in Tissuetek (Fisher Scientific). Besides tissue that was specifically designated for alternative fixation methods, all live tissue was placed in 10% formalin overnight and then cryoprotected in 30% w/v sucrose solution for 24hrs. Following this, tissue was snap frozen in Tissuetek via immersion in -70°C isopentane on dry ice. Frozen sections were cut at 14µm thickness by cryotome (Leica Model CM 1900 3-1, Leica Microsystems).

Alternative fixation methods included overnight fixation in either 4% paraformaldehyde (all SCAP tissue) or 0.5% gluteraldehyde.

3.3.2 Dye preparation

All solvatochromic dyes tested (Laurdan, Prodan, Nile Red, Nile Blue) were prepared from powder into 1mM stock concentrations in DMSO. Stock solutions were kept at 4°C, removed from light. Working dilutions (5,10,20,40,100µM) were prepared immediately before use by mixing stock concentration with dPBS (Gibco ON, Canada).

3.3.3 Tissue staining

Sides were stained by first rehydrating the samples for 10 minutes in dPBS, followed by exposure to a bath of the appropriate dilution of dye, prepared in a stand alone 50ml glass slide

rack. Staining time was exactly 30 minutes, followed by two sequential 10-minute PBS washes, all performed in the dark. Slides were then tapped dry, coverslipped using Fluorosave (Millipore, Billerica, MA, USA), and placed in the dark pending confocal imaging. All images were taken within 2hrs. of staining.

3.3.4 Animals

Adults rats - Adult sciatic nerve tissue was obtained through the sacrifice of 8-week-old adult Male Lewis rats, weight matched from 255-290g. *Post-natal rats* -Developmental sciatic nerve tissue was obtained from Lewis pups, of post-natal days 2, 7, 14, and 28 respectively. *Shiverer mice* - Shiverer mutant (JAX C3Fe.SWV-*Mbp*^{shi}/J) and wildtype (C3HeB/FeJ) sciatic nerve was obtained from adult mice, age matched from Jackson Labs. *SCAP mice* - Sciatic and Optic nerve tissue was obtained from age-matched adult SCAP knockout and control mice (courtesy of Dr. Mark H. G. Verheijen, VU University, Amsterdam) ¹⁵⁶.

3.3.5 Confocal Microscopy

See Chapter 2 “General Methods” for a detailed summary of equipment and confocal microscopy technique. Of note, all images taken for the chapter were obtained at room temperature (24°C).

3.3.6 Spectral Analysis and Processing

See Chapter 2 “General Methods” for an in-depth description of spectral image processing for both mean spectral data collection, as well as for transformation of raw spectral image data into

average spectral peak-by-pixel population data (ASP). Also refer to the methods of obtaining “selection regions” using the mask by spectra tool (Imagetrak, Peter Stys).

Specific to this chapter, analysis of post-natal myelin was performed using the solvatochromic dyes Laurdan and Nile Red. Myelin was analyzed as toroids on axial sections of sciatic nerve. Each dye was analyzed for averaged mean normalized emission signal (amongst collated nerves at each timepoint), as well as averaged spectral peak pixel population (ASP). For the latter method, Nile Red ASP was calculated using bracketing spectra of Nile Red in pure phospholipid (phosphatidyl choline; Red shifted bracket) and Nile Red in pure triglyceride (Blue shifted bracket). This particular ASP for Nile red is designated at the Polarity Index of Nile Red (see NRSSA analysis method, Chapter 2). Index % measurements were achieved by normalizing the X-axis population bin-widths to a percentage-based axis.

Laurdan ASP is reported as such, and no specific “polarity index” bracket was formulated for this dye, due to technical constraints. Instead, the blue-edge bracket spectrum for Laurdan ASP was the computer-generated spectra from shifting the “Adult myelin Laurdan spectra” 20nm to the left. Likewise, the red-edge bracketing spectra were defined as “Laurdan adult spectra +20nm” for developmental myelin, and “Laurdan adult spectra +40nm” for mutant myelin (a longer bracket necessitated by the overall red shifted emission spectra of mouse mutant myelin). Wavelength measurements were designated to bin-widths by normalizing the X-axis divisions, equal to the sum total of the shifted brackets (i.e. 40nm total for the developmental data, 60nm total for the mutant data).

3.4 Statistics.

For Aim 1, differences between two means were calculated using the unpaired student's t-test. Differences between multiple means were calculated using one-way ANOVA, and post-hoc multiple comparisons performed using the Tukey's test.

3.5 Results

3.5.1 Baseline Control Experiments

3.5.1.1 Consistency Controls

Each of four solvatochromic dyes (10 μ M) was used to dye biological replicants of age-matched adult sciatic nerve (Figure 3-1).

Results are shown in Figure 3-2. Both blue dyes demonstrated low standard deviations for peak spectral values amongst replicants (Prodan = 0, Laurdan = 1.2). Of the red dyes, Nile Red displayed the lowest standard deviation (5.4), while Nile Blue displayed a comparatively large standard deviation (14.4). Of note, the standard deviation of the Nile Red group is very low (0.8) when the outlier of 612nm is removed.

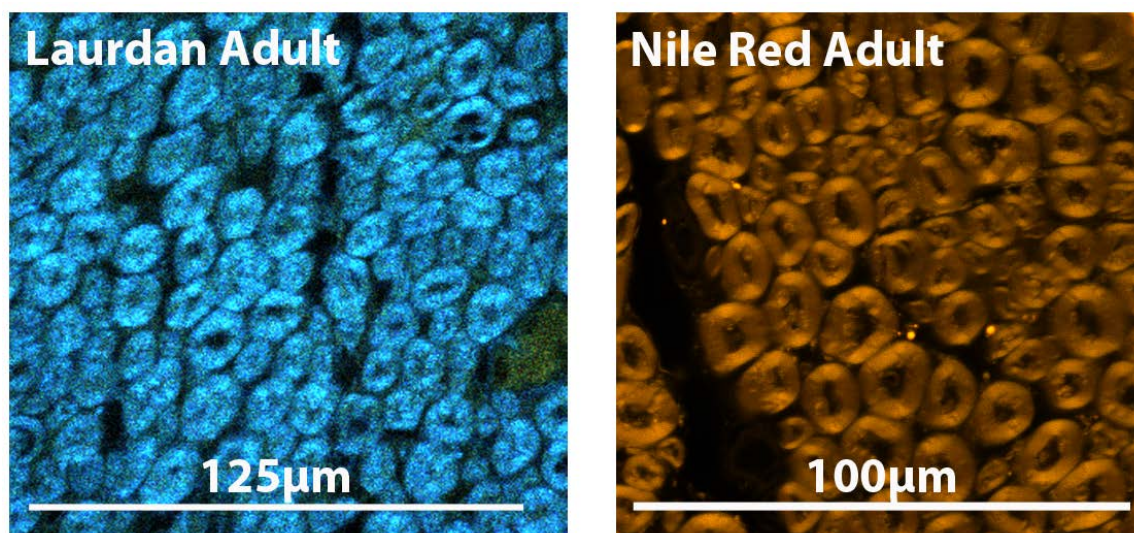


Figure 3-1 High magnification examples of adult rat sciatic nerve. Sciatic axial sections (12µm thickness), stained with 40µM Laurdan and Nile Red respectively.

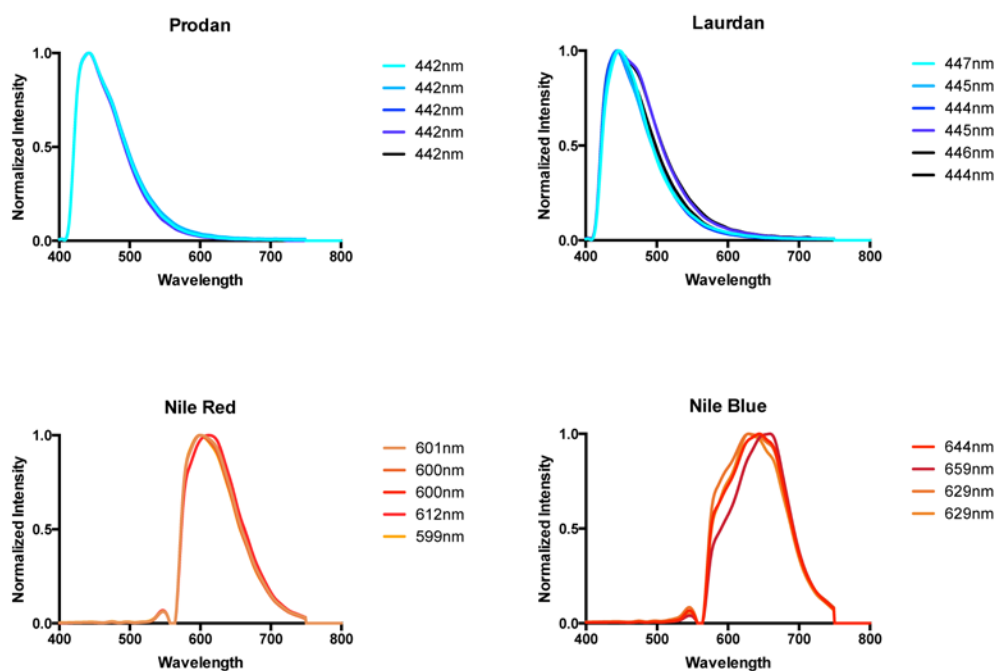


Figure 3-2 Mean spectral analysis of myelin toroids from adult sciatic nerve biological replicants. Prodan, Laurdan, and Nile Red display reliable spectral peak emission signatures

across replicants (SD 0, 1.2, 5.4 respectively), while Nile Blue shows increased variability (SD 14.4).

3.5.1.2 Concentration Controls

As previous work has demonstrated a potential for concentration-induced spectral shifts amongst solvatochromic dyes⁵⁹, we decided to test each dye for this potentially important phenomena. These results are shown in Figure 3-3.

Three of the four dyes tested showed a concentration dependent shift of emission wavelength. Prodan and Nile Red (488nm excitation) demonstrated a blue shifted emission spectra at concentrations greater than 10 μ M. Interestingly, Nile Red excitation with 561nm light abolished this effect until dye concentration approximated 100 μ M. Laurdan also displayed a blue shifted emission at concentrations greater than 20 μ M. Nile Blue emission remained quite variable, with no conclusive effect demonstrated that was proportional to dye concentration.

It should also be noted that it was quite difficult to achieve adequate signal from the blue dyes (signal>tissue autofluorescence) at the lowest concentrations employed (5,10 μ M), particularly with Prodan. Data shown represent multiple repeated attempts at achieving sufficient fluorescence with Prodan staining at concentrations <20 μ M, such that further work with Prodan beyond this preliminary effort was not continued.

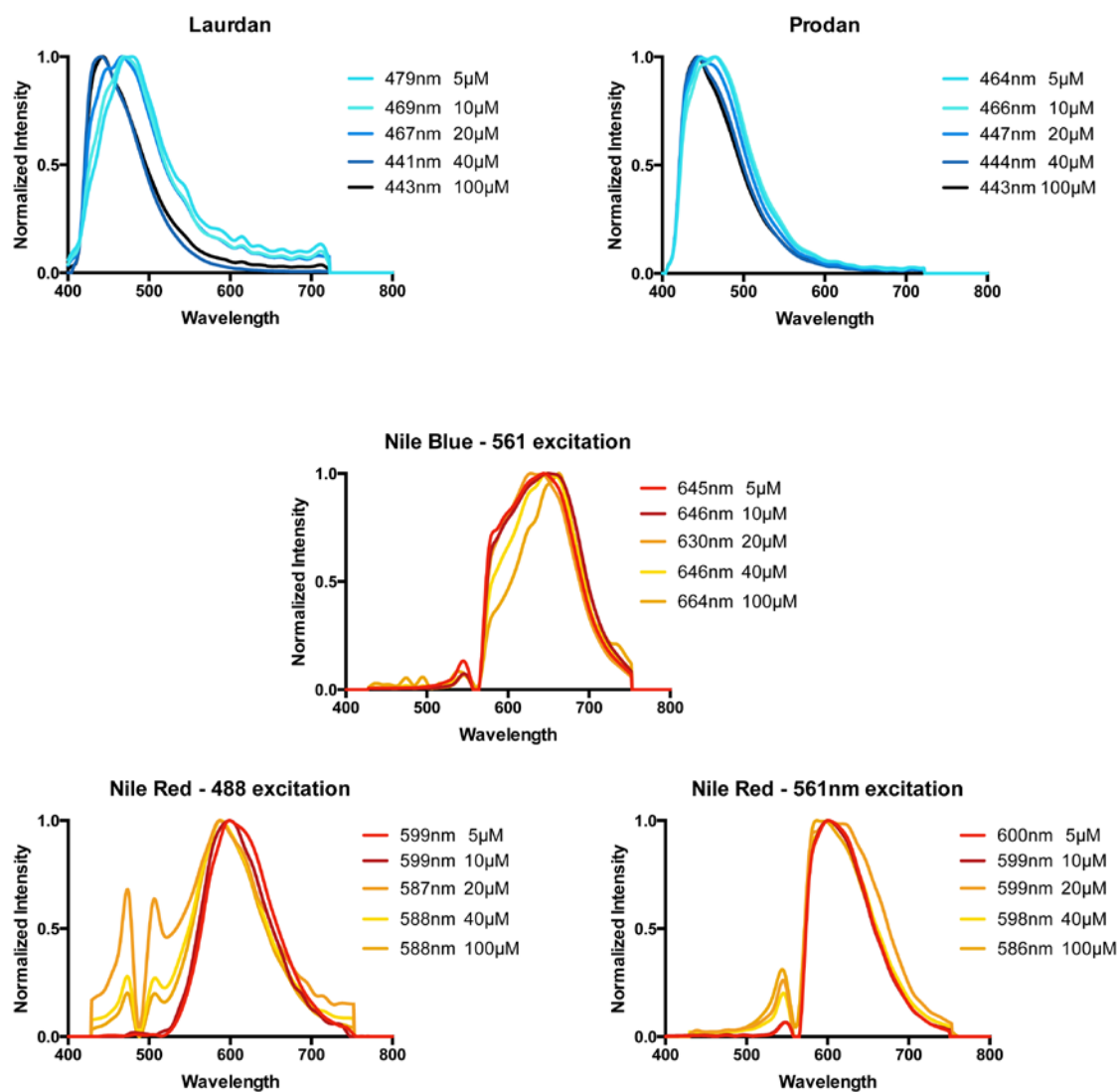


Figure 3-3: Effect of dye concentration on spectral emission in myelin. Laurdan, Prodan, and Nile Red all display blue shifted emission spectral peaks with increasing concentrations of dye employed. Nile Blue emission remains highly variable as previously demonstrated.

3.5.1.3 Effect of Fixative

As common fixatives result in the cross-linking for proteins,⁶⁷, we tested both Nile Red and Laurdan for fixative induced spectral effects in adult sciatic sections (rationale for restriction of analysis to these two dyes will be explained in the following discussion). Our results indicated no discernable effect on emission spectral peak for Nile Red or Laurdan (Figure 3-4).

Conditions tested included gluteraldehyde fixation, no fixation (fresh nerve) with and without overnight 30% sucrose immersion for cyroprotection, as well as 10% formalin fixation. The SD of emission for each dye across fixation methods (Laurdan, SD 1.26; Nile Red SD 1.26) was comparable to emission SD previously demonstrated across biological replicants.

One notable difference amongst fixative is that gluteraldehyde fixation tends to result in an increased background signal, as evidenced by the greater intensity of the red emission tail in Laurdan emission spectra. Of all fixation methods examined, 10% formalin fixation results in the least background fluorescence signal.

3.5.2 *Efficacy Experiments*

3.5.2.1 Spectral analysis of post-natal myelin (Nile Red, Laurdan)

3.5.2.1.1 Laurdan - Laurdan (10 μ M) mean spectral analysis revealed a biphasic spectral evolution with increasing postnatal age.

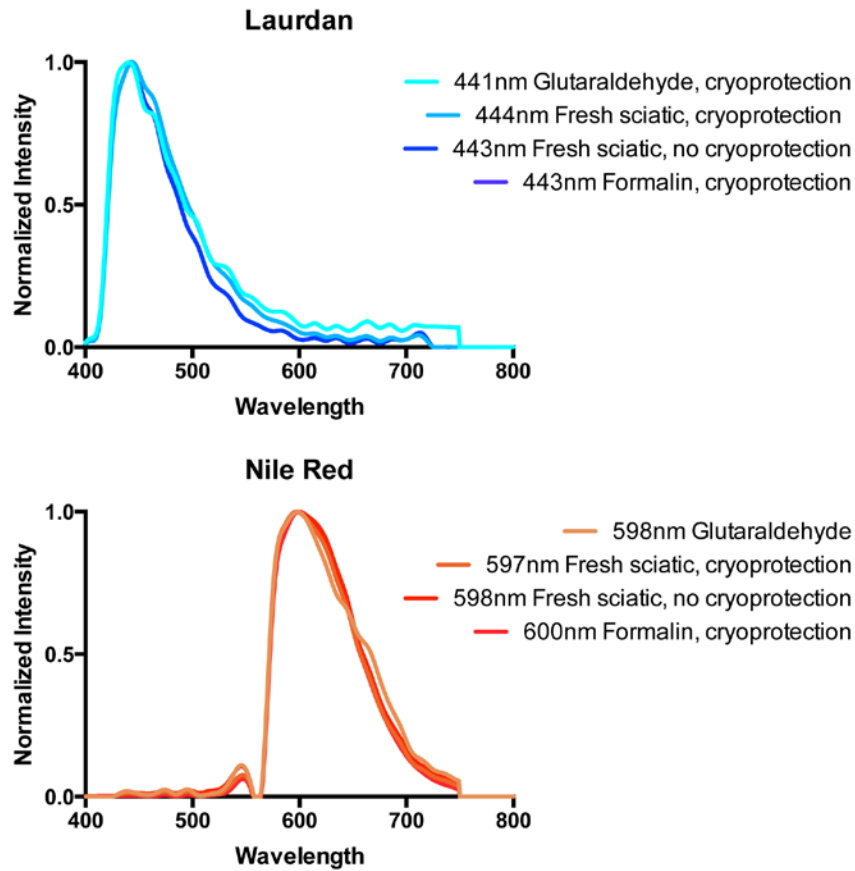


Figure 3-4 Effect of fixative on spectral emission in myelin. 10 μ M Laurdan stained sciatic nerve demonstrates minimal variance across fixation styles (SD 1.26), as does sciatic nerve stained with 10 μ M Nile Red (SD 1.26).

P3 myelin (n=4) was relatively blue shifted (452 $\pm$ 6.2nm peak) compared to other post-natal time points, while demonstrating a red-shifted spectral “shoulder”. P7 (n=5) and P14 (n=4) myelin showed red-shifted spectral peaks (466 $\pm$ 8nm, 479 $\pm$ 5.7nm), both with blue shifted spectral shoulders (P7<P14). P22 (n=4) myelin (445 $\pm$ 0.3nm peak) was most similar to adult myelin (n=6) (444 $\pm$ 0.4nm), with only a small red-shifted shoulder (figure 3-5a).

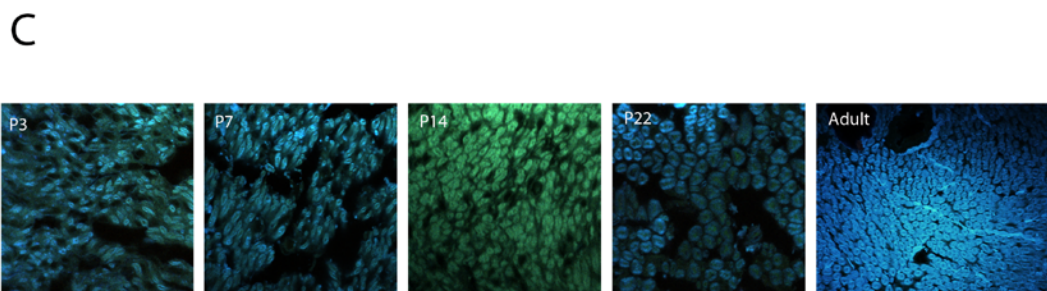
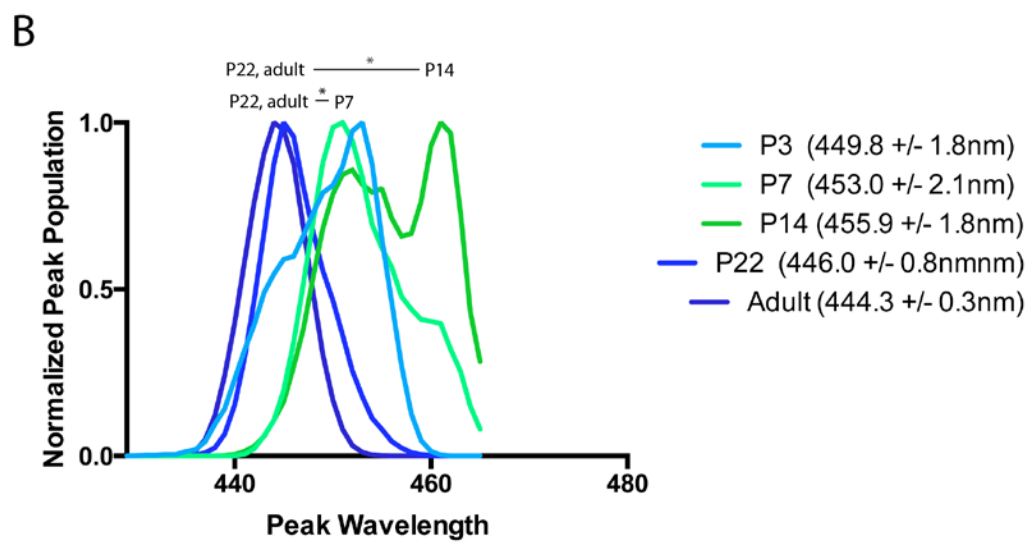
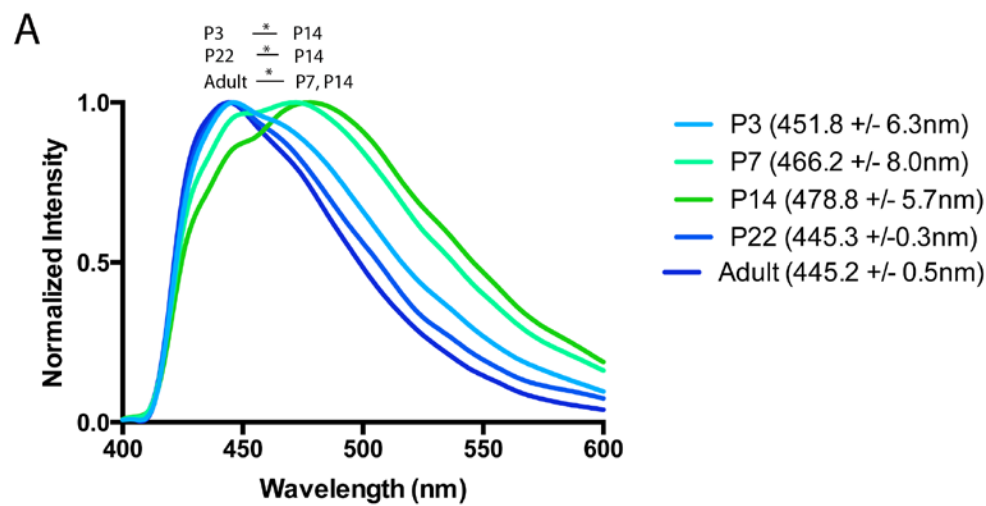


Figure 3-5 Laurdan analysis of post natal sciatic nerve myelin. A) Mean emission spectra of post-natal rat sciatic nerve, 10 μ M Laurdan. *represents $p < 0.05$ between groups, one-way ANOVA, Tukey's post test. Mean emission spectra demonstrate two primary emission states. B) However, Average spectral peak population analysis demonstrates three distinct populations of spectral peak emission by pixel, for developmental epochs. C) Representative screen shots of true color images for each of P3, P7, P14, P22, and adult sciatic nerve myelin, stained with Laurdan.

These differences reached statistical significance (one way ANOVA, $p < 0.05$, Tukey's post-hoc test) for the following pairings: P3 and P14; P7 and Adult; P14 and P22, Adult.

Laurdan ASP analysis demonstrated three populations of Laurdan spectral emission; P3 ($n=5$, 450 $\pm$ 1.9nm) and P7 ($n=5$, 453 $\pm$ 2.1nm) myelin displayed primarily populations of an intermediate emission spectral peak (figure 3-5b). P14 myelin ($n=8$, 456 $\pm$ 1.8nm) demonstrated the most pronounced red shifted population, while P22 myelin ($n=8$, 446 $\pm$ 0.7nm) was similar to adult myelin ($n=4$, 444 $\pm$ 0.3nm). Differences between these groups reached statistical significance for the following pairings (one way ANOVA, $p < 0.05$, Tukey's post-hoc test): P7 and Adult; P14 and P22, Adult.

3.5.2.1.2 Nile Red

Nile Red mean spectral analysis (figure 6a) demonstrated progressively blue shifted spectra with increasing developmental age: P3, P7 < P14, P22, adult ($p < 0.05$, one-way ANOVA, Tukey's post-

test). Average emission wavelength were as follows: P3 (n=12, 606 +/- 1.2nm); P7 (n=8, 607 +/- 1.8nm); P14 (n=8, 598 +/- 1.4nm); P22 (n=7, 600 +/-1.0nm); Adult (n=6, 596 +/- 0.8nm).

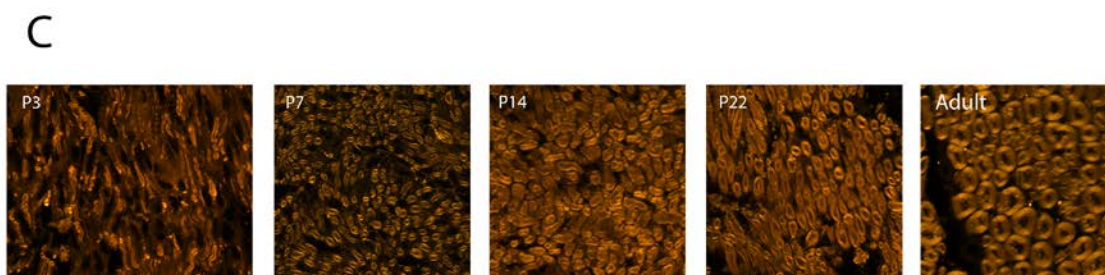
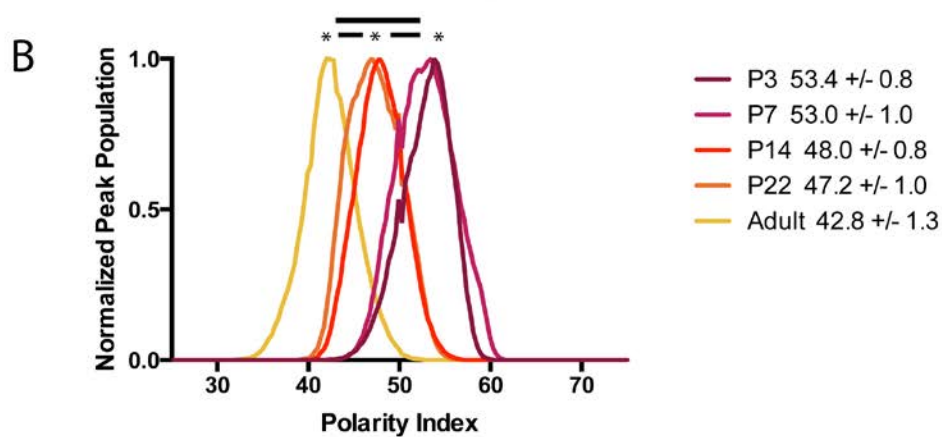
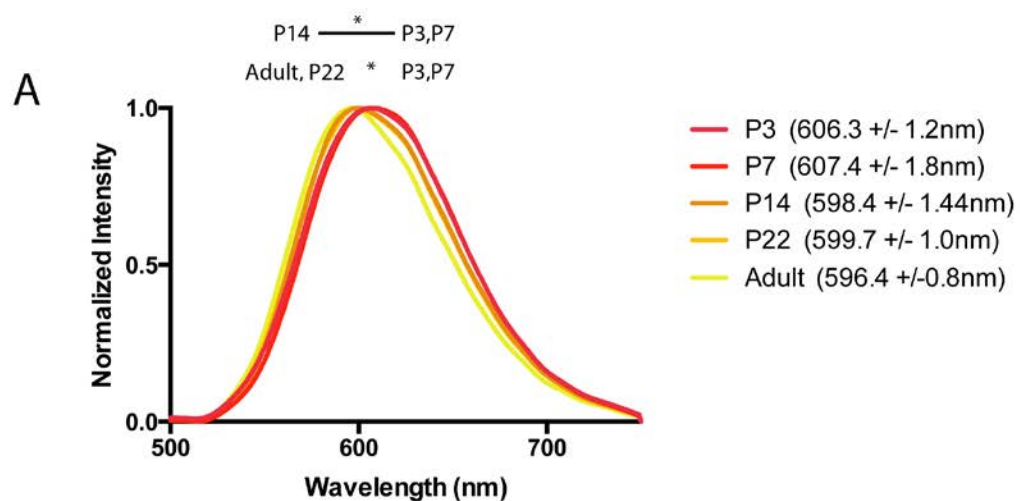


Figure 3-6 Nile Red analysis of post natal sciatic nerve myelin A) Mean spectral emissions of post-natal rodent sciatic nerve (P3, P7, P14, 22, stained with 10 μ M Nile Red. B) Polarity Index of post-natal myelin stained with 10 μ M Nile Red. C) Representative screenshots of analyzed nerve fields for each age grouping. *indicates significance difference between mean ($p < 0.04$, one-way ANOVA, Tukey's post test).

Nile Red ASP (polarity index) analysis (figure 6b) demonstrated a similar trend (P3,P7 < P14,P22 < adult, < 0.05 , one way ANOVA, Tukey's post-test). Specific PI averages were as follows: P3 (n=8, 53.4 $\pm$ 0.8); P7 (n=8, 53.0 $\pm$ 1.0); P14 (n=8, 48.0 $\pm$ 0.8); P22 (47.3 $\pm$ 1.0); Adult (42.8 $\pm$ 1.3).

3.5.2.2 Spectral Analysis of Mutant Myelin (Nile Red, Laurdan)

3.5.2.2.1 Laurdan + Shiverer

Mean spectral emission of Laurdan (10 μ M) demonstrated only non-significant differences (unpaired t-test) between peak emission wavelength amongst Shiverer (n=7, 491 $\pm$ 0.4nm) and wildtype control nerves (n=7, 489 $\pm$ 1.0) (Figure 3-7a). Laurdan ASP analysis did however show a significant difference between mutant (n=7, 493 $\pm$ 1.4nm) and wildtype (n=7, 500 $\pm$ 0.8nm) peak emission populations (Figure 3-7b).

3.5.2.2.2 Laurdan + SCAP

Laurdan was ineffective at reporting any difference between SCAP knockout and wildtype sciatic nerves.

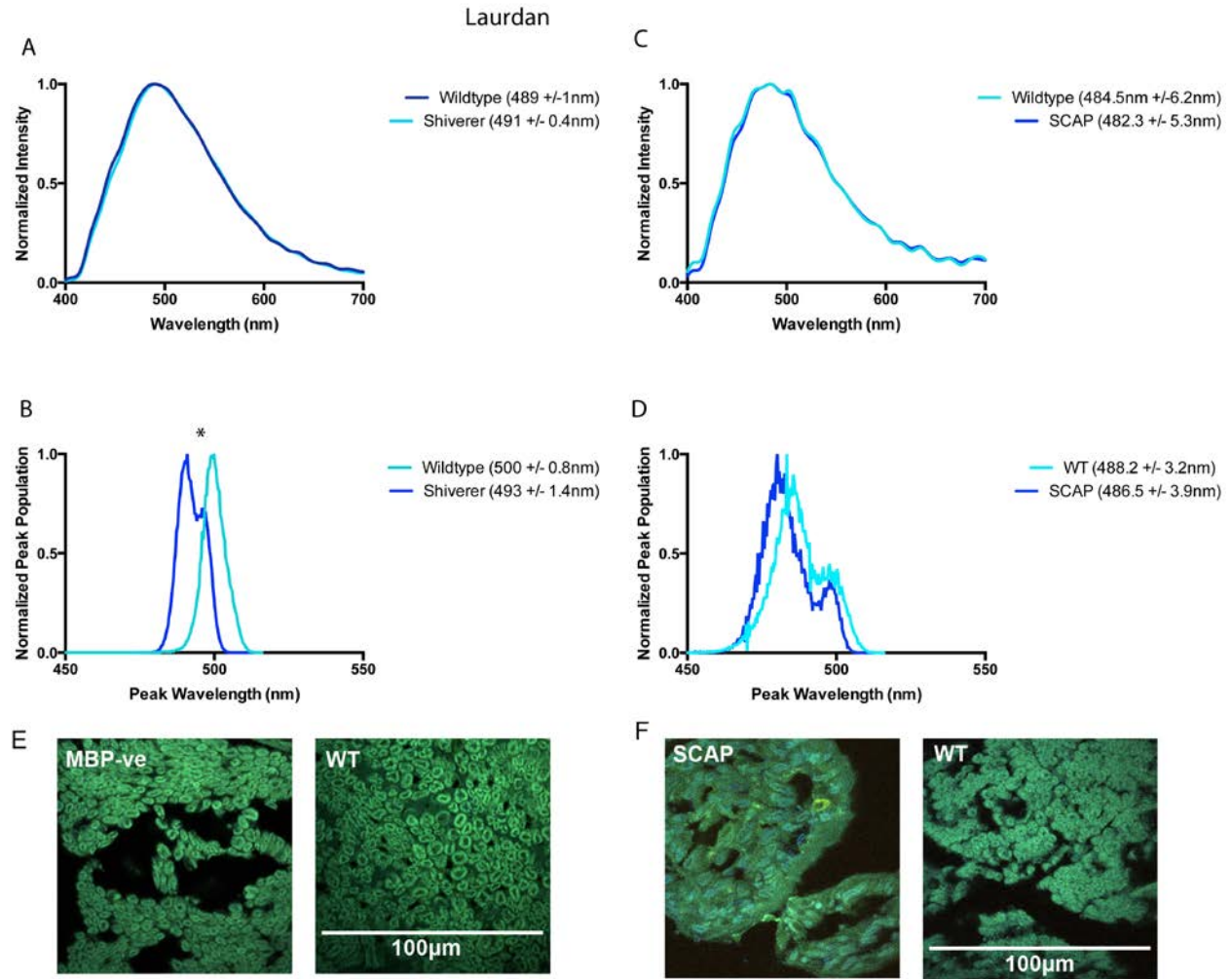


Figure 3-7 Laurdan analysis of mutant myelin A) Mean average spectral emission peak for Laurdan (10 μ M) stained Shiverer vs. Wildtype mouse sciatic nerve are very similar, though B) Laurdan ASP analysis did demonstrate a significant difference ($p < 0.005$, paired t-test) between the labeled spectral populations. C) Neither mean spectral or D) ASP analysis of Laurdan stained SCAP vs. Wildtype sciatic nerve revealed significant spectral differences. E) Screenshots of Shiverer vs. Shi Wildtype sciatic nerve. F) Screenshots of SCAP vs. respective WT sciatic nerve.

Mean spectral analysis showed mean emission of SCAP nerves (n=4) at 482 +/- 5.3nm, and wildtype (n=6) nerves at 485 +/- 6.2nm, for a p- value of 0.81 (unpaired t-test) (Figure 3-7c). Laurdan ASP analysis of SCAP (n=4, 486 +/- 3.9nm) and wildtype (n=6, 488 +/- 3.2nm) was also not significant for differences (p=0.74, unpaired t-test) (Figure 3-7d).

3.5.2.2.3 Nile Red + Shiverer

Nile Red (10 μ M) indicated a significant difference in spectral mean emission and polarity index between Shiverer (n= 8) and wildtype controls (n=7). Shiverer mean average peak emission was 603 +/- 0.2nm; wildtype 600 +/- 0.2, and significant at p <0.0001 (unpaired t-test) (Figure 3-8c). Shiverer Nile Red PI was 50.4 +/- 0.3 (n=8), while wildtype PI was 47.7 +/- 0.4 (n=7) (p<0.001, unpaired t-test) (Figure 3-8d).

3.5.2.2.4 Nile Red + SCAP

Nile red (10 μ M) analysis of SCAP (n=4) and control wildtype (n=4) nerves did not display a significant difference (p=0.3) in mean spectral peak emissions (SCAP 597 +/- 3.8nm; WT 593 +/- 1.0nm) (Figure 3-8a). Solvent polarity analysis did however reveal an overall difference in peak spectral emissions populating the selection region, with SCAP myelin showing a SP of 42.0 +/- 1.4, as compared to the SP of 36.9 +/- 1.5 for wildtype control myelin (p<0.05 unpaired t-test) (Figure 3-8b).

3.6 Discussion

3.6.1 Spectral Analysis of Experimental Controls

3.6.1.1 Dye emission is stable across biological replicants

Reliable spectral emission signatures were achieved for Prodan, Laurdan, and Nile Red, across anywhere from 4-6 biological replicant sciatic nerves from adult age controlled rats. Nile Blue however demonstrated a peak emission that varied across 30nm of emission spectrum. To explain this we consider that Nile Blue may undergo conversion to Aurmanine 0¹⁵⁸, a dye of yellow emission fluorescence, though this reaction is usually catalyst induced. However, early synthesis of Nile Red was accomplished by hydrolysis of Nile Blue⁷⁸, also suggesting an inherent instability of Nile Blue in solution.

We observed a general blue shifted emission of Nile Blue myelin spectra with increasing time after staining, as little as a few hours (data not shown). Taken together, we suggest that Nile Blue is unreliable for the purpose of reporting spectral information from a biological membrane such as myelin, using the techniques available to us.

3.6.1.2 Spectral emission is concentration dependent

Our data suggest that fluorescence emission undergoes a blue shift once a certain “threshold” concentration value is reached, which is dye dependent. The thresholds we have seen are as low as 10 μ M (Prodan), and as large as 100 μ M (Nile Red – 561nm excitation). Concentration dependent fluorescence has been demonstrated previously for Nile Red⁵⁹, revealing a red-shifted spectra with increasing concentrations.

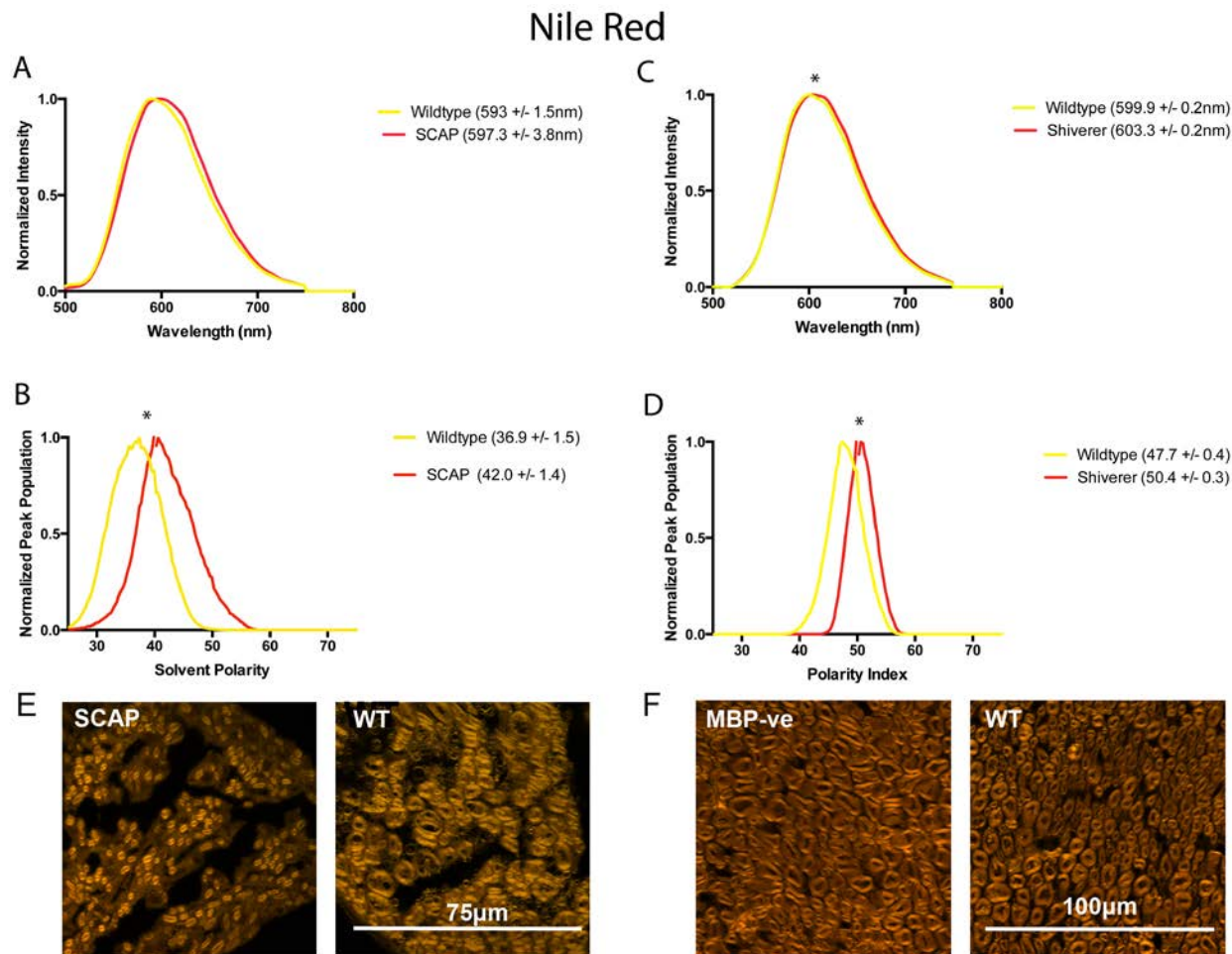


Figure 3-8 Nile Red analysis of mutant myelin. A) Nile red mean emission peaks were not statistically different for SCAP vs. WT control myelin, though B) polarity index analysis demonstrated did reveal significant differences in the overall spectral populations ($p < 0.05$, unpaired t-test). C) Likewise, Nile Red mean spectral analysis demonstrated significant emission differences between Wildtype and Shiverer mutant myelin ($p < 0.0001$, unpaired t-test), as well as D) spectral population differences as demonstrated by polarity index measurement ($p < 0.001$,

unpaired t-test). E) Screenshots of SCAP and respective wildtype sciatic nerve. F) Screenshots of Shiverer and respective wildtype sciatic nerve.

However, as the highest concentration employed in that study was approximately 0.1 μ M (50x lower than our lowest concentration used), it is difficult to compare this data with our own.

It is possible that after a certain threshold value, the probability of dye molecules interacting with themselves increases to the point where they reliably affect each other's solvent sphere. Alternatively, it is possible that dye saturation may result in displacement of dye molecules to other membrane locations, perhaps towards the lipophilic core. Likewise, proximity affects might encourage conformational changes in dye molecules (e.g. Planar vs. Twisted) that may result in different emission spectra⁶⁴.

3.6.1.3 Myelin spectral emission is not dependent on fixation or fixative type

We examined both Laurdan and Nile Red for effects of formalin and gluteraldehyde fixation on emission spectra. Somewhat surprisingly there seemed to be no effect of fixative type on emission spectra, nor any effect of cyroprotection with overnight immersion of tissue in 30% sucrose. Finally, the effect of fixation in and of itself did not seem to alter emission spectra vs. fresh stained tissue. We can comment that the effect of protein-cross linking on dead tissue is not detectable by our current methods, using these dyes.

3.6.2 Myelin development proceeds with biochemical changes that can be interrogated by solvatochromic dyes

Analysis of the myelin of early development was chosen as a testing ground of sorts, to help determine the efficacy of our solvatochromic dyes to report on known changes to the chemistry and structure of rat sciatic nerve myelin over the first month of life^{18,48,71,143}. In this respect, both Laurdan and Nile Red revealed a strong potential capacity for reporting on these differences.

Our data suggests that the process of early developmental myelination proceeds with three distinct phases of chemical environment, as revealed by the three distinct spectral populations demonstrated by both Nile red and Laurdan (figure/s). These populations suggest transitions in the chemical environment of myelin, particularly between Day 7 to Day 14 of postnatal development, through to day 22, when the environment detected by Laurdan becomes similar to controls. Nile Red shows a markedly similar grouping, except that at P22, the environment detected by Nile Red is not yet similar to adult myelin. This is one indication that these dyes are likely indicating different membrane environments

Indeed, a major implication of this data is that Nile Red and Laurdan are exposed to different “solvent spheres”, at least in regards to their integration into developmental myelin, and are likely situated at different regions of the plasma membrane. It is known that Laurdan is very likely incorporated at the outer region of the membrane bilayer, adjacent or even superficial to the phospholipid groups, and in this position is likely to interact primarily with water molecules as it’s relevant solvent sphere¹²⁰. Nile Red, on the other hand, binds at the interfacial region¹¹².

It likely has direct interactions with the polar head groups, and has primarily been described as a probe sensitive to changes in lipid membrane order as opposed to hydration status⁸⁹

3.6.2.1 Laurdan

The emission wavelength of Laurdan is commonly associated with membrane hydration status, so much so that the two primary emission states of Laurdan in bilayers are termed “solvent (water) relaxed” (490nm emission), and “solvent non-relaxed” (440nm emission)^{32,157}. The mean spectral emission wavelength of Laurdan-stained developmental tissue roughly corresponds to one of these two states in our experiment (figure 5a). However, it has also been demonstrated that Laurdan will also demonstrate emission shifts proportional to lateral membrane packing density as well¹²⁰. This notion is also suggested by the data of De Vequi-Suplicy et. al ³², whereby DLPC vesicles present with an intermediate Laurdan emission spectrum as they are progressively warmed. It is commonly thought that these lipid density detecting shifts are also mediated by water interactions, as tightly packed membranes have lesser access to water interaction, and hence encourage Laurdan emission in the “solvent non-relaxed” state⁴.

It is possible that any intermediate emission spectra is the simple summation of varying ratios of emission from the two previously mentioned Laurdan hydration states. Our data suggests that Laurdan emission (in developmental nerve tissue at least) indeed exists in a third population whose peak value is of intermediary energy (figure 5b). Laurdan is in fact known to have a third, less common emission energy¹⁵⁷, though it is only present in extremely cold aprotic solvents, and has a blue-shifted emission wavelength of 400nm. If we accept our intermediate

peak population of emission as real, we may be seeing low intensity emission phenomena that may be commonly masked by the high intensity emission of the two main solvatochromic states for Laurdan. This emission state would also present a mathematical solution for the observed “blue shifted” and “red-shifted” shoulders commonly seen with Laurdan spectra.

It is likely, however, that this population (P3, P7) appears for a different reason. Emission spectra for Laurdan in most every solvent condition have been extensively worked out¹⁵⁷, and it would be unusual for developmental myelin to present with such a unique chemical environment to as to provoke a previously undocumented emission energy state for Laurdan. Considering that the averaged spectra of the solvent-relaxed and solvent non-relaxed emissions may summate to a peak value somewhere in-between the two pure emission curves, we may be demonstrating simply that young myelin presents in a state of mixed membrane hydration. As the myelin matures (P14), a greater proportion of membrane bound Laurdan is exposed to water, which may signify a greater presence of lipid disordered myelin lamella that have yet to compact laterally. In support of this theory is the fact that P14 represents the pinnacle for the rate of myelin formation by volume in the rat sciatic nerve. The later time points (P22, adult) present with the non-hydrated emission state, perhaps reflecting efficient compaction of the myelin membrane and greater exclusion of water molecules from the intra-myelin environment. We therefore feel that Laurdan may be an efficacious probe for determination of myelin compaction, through detection of presence or absence of membrane hydration.

3.6.2.2 Nile Red

In contrast to Laurdan, Nile red interacts at the membrane in the interfacial region, deep to the head-groups but superficial to the long hydrocarbon interior¹¹². In addition, Nile red would seem to have no fixed emission states, but varies its emission properties dependent on factors such as membrane polarity¹⁷⁷, cholesterol content¹¹², and membrane lipid order⁸⁹.

Of these factors, our results are consistent with what is known regarding both cholesterol content and lipid order of myelin membrane with increasing developmental age. We show that with increasing post-natal age, myelin displays a progressive blue shifted population of Nile red spectral peaks (Figure 5B). Sciatic nerve cholesterol content in myelin increases steadily with post-natal age¹⁷⁸, which itself may account for some degree of membrane lipid order increase⁷⁹. However, the degree of expression of myelin compaction proteins (e.g. P0 protein) is also increasing over these first weeks of post-natal life¹⁴³. Many of these proteins including P0 are trans-membrane¹⁴¹. While they no-doubt contribute to the lamellar compaction and ultrastructural stability of myelin membrane¹⁴², it is likely that they also contribute towards increased lateral membrane organization as well⁶⁹ by association with cholesterol and glycosphingolipid enriched microdomains. Conversely, it seems that targeting of P0 protein from the ER to the myelin membrane is a cholesterol dependent process¹³⁶, so it is very likely that Nile Red may be sensing a complex interplay between all of these factors, representing a blue-shifted emission spectra with increasing lipid order, cholesterol content, and trans-membrane incorporation of myelin compacting proteins.

We also know that the lipid fatty acyl components of the myelin sheath evolve in early development, and move from a population containing large amounts of shorter hydrocarbon chains (C16:0) to an adult population containing longer hydrocarbon chains, primarily oleic acid (C18:1(n-9))⁵⁰. It is known that Nile Red is mostly insensitive to increased hydrocarbon chain length in experimental membranes, and if anything displays a red shifted emission energy as hydrocarbon chain length increases¹⁷⁷. Our results would indicate that neither this effect, nor the potential decrease in membrane lipid order induced by higher proportions of unsaturated fatty acyl chain, is a significant factor contributing to the overall polarity of the myelin microenvironment during developmental PNS myelination.

3.6.3 Mutant and Knockout Myelin

The main finding from our experiments was that both dyes displayed some level of sensitivity to the known chemical and physical alterations in the myelin membrane of both Shiverer and SCAP PNS myelin, as compared to their respective wildtype controls. However, we can see from figures 3-7 and 3-8 that Nile Red is the more sensitive dye in this regard, especially when we employ the average peak spectral population analysis (or polarity index analysis, as established for Nile Red). In particular, PI analysis of Nile Red is sensitive to the chemical differences between adult SCAP KO myelin and wildtype myelin, as detailed in the introduction, while Laurdan ASP analysis is not.

One main difference between SCAP and WT nerve concerns altered cholesterol levels, and by proxy the degree of lateral membrane organization. SCAP adult myelin is comparatively deficient in both parameters¹⁵⁶. Laurdan literature has previously demonstrated extensive use of

this dye to indicate changes in lipid order^{3,4,120}. Why then would our Laurdan analysis fail to detect these known differences? One reason may be that the previous Laurdan studies mentioned were performed in either single membrane layer thick, or single bilayer thick membrane systems. The mechanism of Laurdan's sensitivity to lipid order is dependent on varying degrees of hydration interactions¹²⁰, related to its solvent sphere positioned at the outer extremes of the membrane. It is possible that adult myelin, a compact and multilamellar system (as opposed to developing myelin), may be exclusive enough to water molecules that this "by proxy" ability of Laurdan to comment on lipid order through hydration interactions is lost.

3.6.3.1 SCAP

To elaborate on the above discussion, the effect of cholesterol on lipid order has been investigated in model membrane systems using Prodan and Nile Red^{88,89}. Interestingly, cholesterol has a stabilizing effect on biological membranes. It will increase the polarity and movement of a gel-phase membrane by permeating it and increasing its mobility, however it will decrease these factors for a liquid crystalize membrane (such as myelin). This results in increased order and closer packing for biological membranes. In pure model membrane systems, highly ordered large unilamellar vesicles (LUVs) composed entirely of highly ordered "lipid raft" material (sphingomyelin and cholesterol) demonstrate blue shifted Laurdan emission spectra, as compared to model membranes consisting mainly of palmitoyl-oleoly/dioleoylphosphatidyl phospholipids, representative of the lipid disordered membrane state⁷⁹. Nile Red as well has demonstrated sensitivity to increasing lipid order via progressive cholesterol addition in unilamellar vesicle systems⁸⁹ From this, we also see that lipid order and lipid composition are

highly intertwined variables that depend heavily on one another⁷⁹, and it may be unreasonable to comment that solvatochromic dyes may be reporting on only one of these aspects.

Our results demonstrate that SCAP sciatic nerve, stained with Nile Red, emits with a further red-shifted emission than wildtype controls. This is encouraging as further supportive data towards the effectiveness of Nile Red as a indicator towards lipid composition differences in adult nerve tissue, and is suggestive that Nile Red can be an effective indicator of lipid order changes in myelin.

3.6.3.2 Shiverer

Our results indicate that Shiverer myelin presents with an overall red-shifted spectral population as compared to Wildtype controls. One simple explanation for this observation is that the absence of MBP (a highly cationic protein⁵) may leave the cytosolic membrane with an altered net charge¹⁵. Proteolipid protein is a CNS transmembrane protein that is also positively charged and may likewise contribute towards establishing polarity of the myelin membrane¹⁶⁶; however, while PLP is indeed expressed in Schwann cells, it is not present in the PNS myelin membrane⁸⁰. P0 protein is also cationic and present in the PNS myelin membrane, though perhaps it may be insufficient to fully compensate for the lack of MBP. This increase in overall net negative charge may influence the solvent sphere of Nile Red to emit in a red-shifted fashion. Alternatively, the decrease in positive inner membrane charge may allow Nile Red to migrate closer towards the membrane surface than would otherwise be possible. This may plausibly increase Nile Red interaction with polar head-group moieties, and result in red-shifted emission spectra from a stabilized excited state.

Another potential explanation for the demonstrated high polarity index of Shiverer myelin as compared to wildtype myelin also concerns the concept of lateral membrane density. MBP, at least in the central nervous system, has been shown to influence and in fact is necessary for the aggregation of lipid raft clustering in oligodendrocytes⁴². Though not the main conclusion of the manuscript, they found that oligodendrocytes cultured from Shiverer mice have less demonstrated lipid clustering than wildtype cells, as measured by GalC immunofluorescence. One of the methodologies included the observation of blue-shifted Laurdan emission, as a read-out of increased lipid order. As mentioned previously, this technique has been well established for single vesicle model membrane systems³, in which Laurdan will display a fluorescence emission shift from green to blue when dissolved in a lipid disordered vs. a lipid ordered phase.

If MBP holds a similar role in the peripheral nervous system, one would also expect that Shiverer PNS myelin may display a decreased lipid order as compared to wildtypes, which would be reflected by a red-shifted spectral population as compared to wildtypes. Our Nile Red data is congruent with this, and are suggestive of a similar role for MBP in peripheral nerve myelin membrane organization.

3.7 Conclusions

We have determined in a systematic fashion the superiority of Nile Red as a solvatochromic fluorophore for use in labeling peripheral nerve myelin, having the strongest potential of the four

dyes tested to reliably indicate the chemical changes that may be occurring in myelin as it proceeds from early to late myelination.

With that specific goal in mind, Nile Red showed the essential quality of reliable and stable emission spectra over repeated biological replicants of adult myelin. As we demonstrated a concentration-induced effect of these dyes (at concentrations larger than 10 μ M), Nile Red proved sufficiently bright to enable continued analysis within this constraint. Further experiments would be conducted only at concentrations of 10 μ M exactly. In addition to obtaining this valuable parameter, we also were able to appreciate the lack of effect that fixation has on emission spectra. Although 10% formalin is our standard tissue fixative in this experiment, this information was also useful as a baseline control.

Nile Red proved a superior indicator of PNS myelin composition. It was sensitive to developmental stages of myelin formation in the rat sciatic nerve, and also able to indicate the aberrant myelin composition of mutant and knock out mice. Laurdan, though effective for looking at developmental myelin, was comparatively insensitive to the altered chemistry of adult mutant myelin.

As we considered the technical challenges going forward, especially for the goal of live spectral imaging, Nile Red usage also made sense from a technical standpoint. Imaging live axons in-vitro and in-vivo requires the use of inherently fluorescent axon tissue. For the most part, these engineered neurons are designed with green or yellow emitting fluorophores. Considering the low quantum yield of Prodan and Laurdan at concentrations <10 μ M, there is a big concern that dye emission signal would be interfered with by 1) comparatively large G/YFP emission signal,

or 2) laser artefact from G/YFP excitation interfering with red-shifted emission spectra.

Furthermore, our current spectrometer (Hamamatsu, Bridgewater USA) has a blue limit of 400nm for spectral detection. Considering that a 405nm excitation beam also nullifies signal capture from the first 10nm or so of this blue limit, we may potentially miss blue shifted spectral phenomena from dyes such as Laurdan. Although there are undoubtedly ways around each of these technical roadblocks, when we consider the already considerable challenges inherent in live imaging, Nile Red is a far more suitable dye. For the most part, it's spectral emission lies outside of the range of GFP or YFP fluorescence, and we can capture all of its range using our exiting spectrometric capability.

To summarize, the data of this chapter provided strong impetus for the use of Nile Red in future aims, as a reliable and effective dye for the interrogation of peripheral nervous system myelin.

Chapter Four: **Doxorubicin injury for the study of in-vivo remyelination in the PNS.**

4.1 Abstract

To follow the kinetics of transplanted cell induced remyelination in-vivo, we required an appropriate injury model to further our investigation. As the effect of any therapy on axonal regeneration is a confounding variable toward the study of remyelination efficacy¹⁴, we decided to pursue a model of focal demyelination. Intrafascicular injection of doxorubicin provides a reproducible model of demyelination without significant axonal loss⁶⁰. Electrophysiological improvement, secondary to remyelination, starts to occur after approximately 4 to 5 weeks^{37,60}. There is prolonged and extensive loss of endogenous Schwann cells after the first week of injury, leaving a window of several weeks whereby injected Schwann cell (or stem cell-derived Schwann cell) grafts may interact with nude axons in a demyelinated environment. In our own hands, we demonstrate the injury kinetics of doxorubicin-induced demyelination through electrophysiology and detailed morphometric analysis. We show that SKP-SCs, when injected into this injury, can incorporate themselves in-vivo to form mature myelin. As SKP-SCs were pre-labeled with both eGFP and cell tracker DiI, we are also able to specify, observe, and quantify the myelin specifically derived from this population.

(This chapter is derived from previously published work and has been re-formatted to fit thesis guidelines)⁶⁰

4.2 Introduction

Skin derived precursor cells (SKPs) are neural crest progenitor cells, that can attain a Schwann cell-like phenotype through in-vitro techniques (SKP-SCs)^{104,159}. It is known that SKP-SCs can express a myelinating phenotype in co-culture with axons¹⁰³, in Shiverer mutants¹⁰³, and in CNS injury models¹¹. However, the specific morphometry of SKP-SC derived myelination has not been quantified. Do SKP-SCs produce myelin that is morphologically mature, and is the myelination provided by SKP-SCs capable of supporting physiologic electrical conduction? Moreover, how robust is the myelination capacity of SKP-SCs? We aimed to identify the myelin specifically derived from SKP-SCs (using cytoplasmic eGFP labeling by viral transduction, combined with membrane labeling by DiI) and hypothesized that SKP-SCs can produce mature myelin when transplanted into a focal demyelination injury⁶⁰.

4.3 Methods

4.3.1 Culture and eGFP labeling of SKP-SCs and Schwann cells

Please see Chapter 2 for details about these techniques.

4.3.2 Animals

Adult male Lewis rats with an initial weight of 250-290g were used for all experimental groups. Rats received either unilateral or bilateral surgery depending on experimental group.

4.3.3 Doxorubicin Tibial nerve injury

Focal demyelination injury was achieved by Intrafascicular injection of 30 μ L of doxorubicin into the proximal tibial nerves of experimental rats. In detail, animals were induced and maintained under inhalational isofluorane anaesthesia, and all surgeries were performed under sterile conditions. The tibial nerve was exposed through a 1.5 cm mid-thigh muscle splitting incision of the biceps femoris muscle, and identified as the largest branch of the sciatic trifurcation, with a course running deep to the proximal origin of the lateral gastrocnemius. Over one minute, 30 μ L of 12.5 μ g/ml doxorubicin (total dose of 0.38 μ g) in 0.9% NaCl (Braun Medical, Irvine CA) was injected into the proximal tibial nerve, approximately 2mm distal to the sciatic trifurcation using a 28 gauge insulin syringe, with the needle directed distally⁶⁰. The site of injection was marked with an 8-0 nylon stitch for future reference.

4.3.4 Experimental Groups

In animals used to investigate doxorubicin injury kinetics, a single doxorubicin injection procedure was performed at day 0. Otherwise, all surgical procedures were of two stages, with surgery for doxorubicin injection at day 0, and surgery for cell or media injection occurring at day 9. Day 9 was chosen as the injection time point to avoid treatment-cell toxicity as delineated by the accepted half-life of doxorubicin and its metabolites^{9,99,118}; also, as suggested by previous in-vitro SKP-SC myelin protein expression dynamics¹⁰³, as compared to the kinetics of doxorubicin injury recovery, we felt it an early enough time point for SKP-SCs to form mature myelin before the robust onset of the endogenous Schwann cell remyelination response.

CMAP cohort -bilateral doxorubicin injection surgery was performed on day 0. The animals were operated on again at day 9, with all rats receiving injection of 500,000 SKP-SCs in 5µl culture medium (DMEM/F12 3:1) into the right tibial nerve, while simultaneously receiving a media injection of the same volume into the left tibial nerve⁶⁰.

Histological cohort – animals received only unilateral surgeries on the right side. The experimental groups were SKP-SC injection (500,000 cells in 5µL), Schwann cell injection (500,000 cells in 5µL), and media injection (DMEM/F12, 5µL)⁶⁰.

4.3.5 DiI Cell labeling

In addition to being inherently fluorescent through eGFP expression, cells were also labeled with a cell tracking dye that localizes to plasma membranes. Twenty minutes prior to injection, all cells were labeled with Cell Tracker CM-DiI (Invitrogen, Burlington, ON, Canada) according to manufacturer's guidelines and previous protocol¹⁵⁹. Cells were then washed in DMEM three times and kept on ice until injection, when both cell groups were resuspended in a 5µL volume of DMEM/F12 media.

4.3.6 Tissue Harvesting

Histology cohort: tibial nerves were divided into two sections, proximal and distal. The point of division was 4mm distal to the epineurial suture marking the site of initial injection. From this cut-face, the proximal segments were processed for either 8µm axial cryostat sections (for immunohistochemical studies), or for teased nerve fibre immunohistochemistry. The distal sections were processed for semithin sections.

CMAP cohort: Immediately following the final CMAP measurement, all rats were sacrificed, and tibial nerves harvested for morphometric analysis.

4.3.7 Electrophysiology

All electrophysiology was performed using the Viking IV electromyography (EMG) recorder (Nicolet Instruments, Madison, USA). Measurements consisted of the compound motor action potential (CMAP) from the foot plantar muscle group, as outlined by England et al.³⁷.

Stimulation electrodes were placed percutaneously in the sciatic notch or over the tibial nerve at the ankle, with the anode 2mm more distal than the cathode in each circumstance. For recording electrodes, G1 was placed in the foot plantar muscles, while G2 was placed in the second digit of the same foot. Stimulation intensity was supra-maximal and defined as stimulus of 5mA (constant current) greater than the lowest intensity that would produce the maximal CMAP amplitude, taken after a 1-minute rest period. Amplitude was defined as the greatest upward (negative) deflection, as compared to baseline. All measurements were taken under a constant temperature of 37°C, using isoflurane anaesthesia.

4.3.8 Immunohistochemistry

Tissue dedicated for frozen section was immediately placed in 10% formalin (EMD, USA) overnight, then transferred to 30% Sucrose/PBS for 24hrs. We then embedded the samples in Tissue-TeK (Sakura Fintek, USA), froze the samples using immersion in -80°C isopentane, and sectioned at 8µm thickness (Leica Model CM 1900-3-1, Leica Microsystems, Concord, Ontario Canada). All cryostat sections were mounted on standard microscope slides pre-treated with 2%

3-Triethoxysilylpropylamine (TESPA) (Sigma). We collected every 8 μ m section for the first 1mm, followed by every 3rd section for the subsequent 1mm.

For immunostaining, slides containing axial frozen sections were washed for 10 minutes in phosphate buffered saline (PBS), followed by two 10 minute washes of 0.2% Triton in PBS. All samples then underwent overnight incubation with primary antibody in PBS containing 2% Bovine Serum Albumin (Sigma, USA), and 0.2% Triton (J.T.Baker, USA). Primary antibodies used included: mouse monoclonal anti-neurofilament (NF) 160/200 at 1:500 dilution (Sigma, USA); mouse monoclonal anti-NF 68,1:500 (Sigma, USA); and rabbit monoclonal anti-voltage gated sodium channel 1.6 (NaV1.6), 1:200 (Alomone, Israel). Following primary antibody incubation, samples underwent washes again as above, followed by incubation for two hours with secondary antibody. Secondary antibodies used included Alexa 405 (goat anti-rabbit) and Alexa 488 (goat anti-mouse), all at 1:200 dilution (Invitrogen). All samples received a final rinse with PBS for 10 minutes before being allowed to dry.

Following application of secondary antibody, selected slides underwent further processing for the application of either Nile Blue or Nile Red (Sigma, USA). These fluorescent probes reliably label membrane and lipid droplets¹⁷, and preferentially localize to compact myelin in peripheral nerve. Briefly, 10 μ M staining solution of either dye was prepared from 25mM storage aliquots, and exposed to the samples for 1hr (stock concentrations were diluted in dimethyl sulfoxide (DMSO), and the final dilution was in PBS). Following this, slides were washed for 10 minutes in PBS, allowed to dry, and cover-slipped.

4.3.9 Teased Nerve Fibre Preparation

Tibial nerves were harvested and immediately placed in 10% formalin for two hours. Nerves were then carefully stripped of their epineurium under a dissecting microscope with 5-0 jeweller's forceps. Small bundles of fibres were then separated from the main nerve while submerged in PBS, and transferred to slides (2% TESPA coated). Bundles would then be observed using fluorescent microscopy (Olympus BX51) for the presence of SKP-SC DiI positivity. These positive bundles were then further teased into single myelinated axon thick preparations. Immunostaining of these samples proceeded as above, with the addition of an extra pre-treatment for 10 minutes in -20°C acetone.

4.3.10 Histomorphometry

Tissue dedicated for semi-thin section analysis was immediately placed in 2.5% glutaraldehyde buffered in cacodylate (0.025 M) for overnight fixation, post-fixed in 2% osmium tetroxide for 2 hrs, and embedded in Epon¹⁶². 1 µm thick sections were then cut and stained with 0.5% Toluidine Blue. Three fields per section were photographed at high power under light microscopy (400x: Olympus BX51), and analyzed with Image Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD)¹⁶². G-ratios were calculated as the axon diameter/total fibre diameter for a given figure.

Morphometric analysis was also performed on 8µm frozen sections, whereby 200µm x 200µm high definition images were captured using the Nikon C1si confocal microscope (Nikon, Canada). Three high-powered magnification fields per section were captured, digitized, and analyzed as above. Myelin morphometry was clearly evident by Nile Blue stained profiles. DiI-labeled, eGFP producing SKP-SC derived myelin was defined as being Nile Blue labeled myelin

figures that either contained the lipophilic DiI positivity in the myelin rings, or that were surrounded by an eGFP positive cytoplasmic ring, or both. Non-SKP-SC derived myelin was defined as being a Nile Blue labeled myelin figure with the absence of either of the two above features. Frozen section myelin morphometry was also analyzed using ImagePro 5.0. We analyzed a total of 190 SKP-SC myelinated figures over the three samples combined.

4.3.11 Electron microscopy

Tibial nerve tissue, as allocated for electron microscope analysis, was fixed in 2.5% gluteraldehyde in phosphate buffer (0.1M, pH. 7.4) overnight, post-fixed for 2 hours in 1% osmium tetroxide, dehydrated with ethanol and EPON embedded according to the methods of Court et al ³⁰. Ultrathin sections were contrasted with uranyl acetate and lead citrate ³⁰ and examined using a Hitachi 7650 transmission electron microscope.

4.3.12 Confocal Imaging

Details on confocal imaging technique can be found in Chapter 2 (common methods) Confocal Images were captured using the Nikon C1si Confocal Microscope⁹² (Nikon, Canada). All images were captured as 32 channel spectral data sets spanning 320nm, using the spectral detector [multi-anode photomultiplier tube (Hamamatsu, Bridgewater USA)]. Images were saved within the Nikon EzC1 software (Nikon, Canada), then exported to, analyzed and unmixed using ImageTrak (image analysis software written by Peter K Stys <http://www.ucalgary.ca/styslab/imagetrak>, Peter Stys). As relevant to our experiment, spectral analysis allowed for the detection and separation of 4 or more fluorescent signals, and also for the separation of emission wavelength signals that may be similar among fluorophores. Spectral

unmixing was performed by referencing control spectra, which were captured from single fluorophore labeled control samples. The control spectra sets (and control stained tissue) were: Alexa 405 (NF primary/tibial nerve); Alexa 488 (NF primary/tibial nerve); DiI (non-eGFP producing, DiI positive SKP-SC cell smear); GFP (eGFP producing SKP-SC cell smear); Nile Red (tibial nerve); Nile Blue (tibial nerve). In detail, to obtain the DiI control spectrum, SKP-SCs were labeled with DiI as above, placed on a glass slide, mounted with Fluorosave (Millipore, Billerica, MA, USA), and coverslipped. These cells were visualized under high magnification (488 laser excitation), and images captured as a 32 channel spectral data set over 320nm, spanning the breadth of the emission spectra. Using Imagertrak, the labeled cells were highlighted as a selection region, and the resultant spectrum saved and used as the control spectrum for DiI. The GFP control spectrum was obtained in a similar fashion, only using GFP positive SKP-SCs that had not been labeled with DiI. For the myelin dyes Nile Red and Nile blue, 8 μ m thick axial cryosections of normal, uninjured tibial nerve were stained with a 10 μ M solution of either dye, mounted with Fluorosave, coverslipped, and imaged with high magnification spectral confocal microscopy (488 laser excitation for Nile Red, 561 laser excitation for Nile Blue). As above, image data was collected as a 32 channel spectral data set over 320nm, spanning the breadth of the emission spectra. Using Imagertrak, myelin regions were outlined and selected, and the resultant spectra saved and used as the control spectrum for these respective myelin dyes. Alexa 405 and 488 control spectra were obtained in a similar fashion from control uninjured tibial nerve, that had been stained only with anti-neurofilament primary antibody (Sigma, as above), and either Alexa 405 (405 laser excitation) or 488 (488 laser excitation) secondary antibody (Invitrogen, as above); in this case, Imagertrak selection regions reflected axons in cross section. To perform spectral unmixing, the experimental raw

spectral data file was unmixed in Imagetrak using 3 or 4 channel unmixing, by assigning the relevant control spectrum to each channel.

Experimental spectral image excitation lasers were as follows: 405 and 488 excitation lasers were used for imaging samples containing Alexa 405 secondary (Neurofilament), GFP, DiI, and Nile Red; 488 and 561 lasers were used to image samples containing GFP, DiI and Nile Blue. All images were captured using the Nikon high power Plan Apo VC objective (60x/1.40 NA)

4.3.13 Statistics

For the CMAP animal cohort we used a paired t-test to analyze the right vs. left CMAP (SKP-SC injection vs. media) at each time point. Significance was reported as $P < 0.05$. Statistical computation was performed using GraphPrism4 (Graphpad software, La Jolla, USA). For the day 33 EPON histological cohorts, differences between groups was established with the Kruskal-Wallis test, with pair specific significance then determined by Dunn's post-test (significance set at $p < 0.05$). For the day 33 analysis of myelin morphometry by fluorescence, as well as the day 60 EPON morphometry, group means were compared using the Mann-Whitney test.

4.4 Results

4.4.1 Tibial nerve doxorubicin injection produces focal demyelination.

The electrophysiology curve obtained from sciatic notch stimulation closely mirrored the results demonstrated by England et al³⁷. In our experiment, the morphology of tibial nerve stimulated foot plantar CMAPs remained normal, while CMAPs derived from sciatic notch stimulation progressively lost amplitude, beginning at approximately day 9 post-injury (Figure 4-1A).

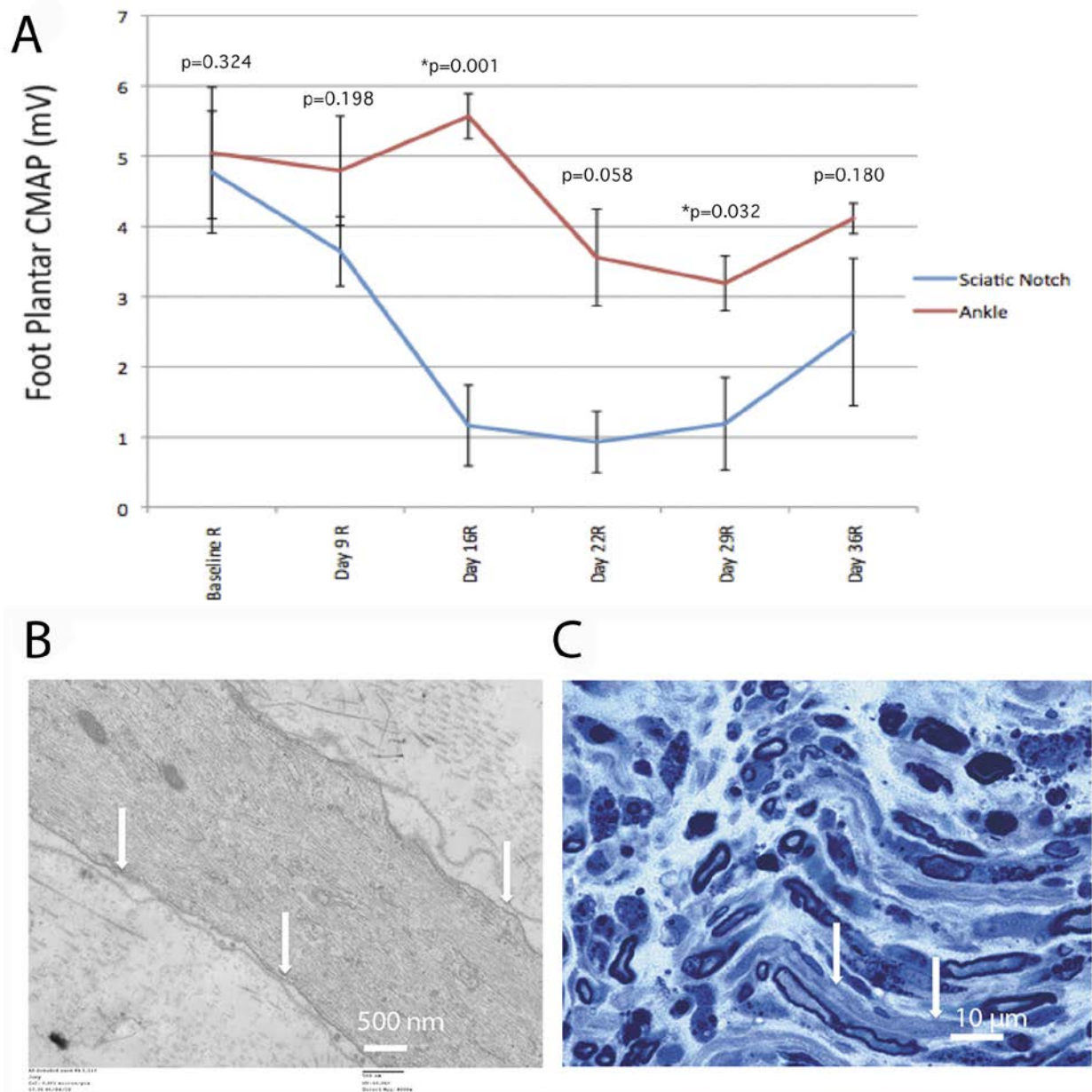


Figure 4-1. Tibial nerve doxorubicin injury. A) Five animals received unilateral tibial nerve injection (30 μ L of 12.5 μ g/ml doxorubicin) at baseline (Day 0). Foot plantar muscle CMAPs were followed over 36 days, with stimulation at both the sciatic notch (blue tracing) and ankle (red tracing) at each time point. CMAPs gradually start to decrease at Day 9 post-doxorubicin, reach a nadir at approximately day 22, and begin to demonstrate partial recovery by day 36.

Ankle stimulation produces CMAPs that remain comparatively unaffected. B) EM (Day 14 post-doxorubicin) showing basal lamina scaffolding (arrows) around a large diameter single axon, denuded of its Schwann cell and myelin sheath, on longitudinal section (scale bar = 500nm). C) 400x magnification image of a longitudinal EPON section, with several axons demonstrating segmental demyelination (arrows) (scale bar = 10 μ m).

These sciatic notch stimulation CMAP also began to display longer latencies and dispersion. Quantitatively, sciatic notch stimulation CMAPs were significantly lower than ankle stimulation CMAPs, at both day 16 (paired t-test $p=0.001$), and day 29 ($p=0.034$), while the day 22 CMAPs demonstrated a strong trend in this regard ($p=0.058$). As well, ankle CMAPs were never significantly different from baseline (Day 9, $p=0.38$; Day 16, $p=0.73$; Day 22, $p=0.42$; Day 29, $p=0.30$; Day 36 $p=0.4285$). Congruently, axon counts (whole nerve NF+ve profile numbers) of tibial nerves injured by doxorubicin injection revealed no significant axonal loss after both day 11 (t-test vs. control, $p = 0.76$) and day 19 ($p = 0.75$). These time points were chosen to investigate the axon counts directly after the initial onset of CMAP abnormality, and during the nadir of CMAP depression, respectively. Electron microscopy (EM) studies, performed during the negative slope of CMAP loss (Day 14), confirmed the separation of Schwann cell basal lamina from large, intact, denuded axons. (Figure 4-1B and 4-1C).

4.4.2 Transplantation of SKP-SCs improves the electrophysiological recovery of doxorubicin injury.

Our results from the paired electrophysiological analysis (Figure 4-2) demonstrated that the SKP-SC treatment group was statistically similar to the doxorubicin group in terms of baseline CMAP measurements and the response of both groups to injury (Baseline – Day 21 no significant difference between groups, paired t-test). The right-sided SKP-SC treated nerves then demonstrated a trend toward faster CMAP recovery at day 27 and 33, finally reaching significance at day 48 post-injury ($p=0.03$). However, at study termination (Day 57), the CMAPs between the two groups were similar, with the left-sided media treatment CMAPs having caught up to the SKP-SC treated right side.

4.4.3 SKP-SCs myelinate axons and participate in mature Node of Ranvier formation.

After sacrifice at post-injury day 33, analysis of 8 μ m thick axial cryosections through the area of cell injection demonstrated strong evidence of DiI labeled, eGFP producing SKP-SCs myelinating axons; these axons were often of large calibre (Figure 4-3). When myelin was stained using a compact myelin dye (Nile Red), the DiI co-localized with the compact myelin of these figures (Figure 4-3).

This was interesting, demonstrating that the DiI labeled phospholipid membrane of injected SKP-SCs does indeed become incorporated into the myelin structure, once these cells attain a myelinating phenotype. Not all of the figures were double-labeled for GFP and DiI. In instances, DiI positive myelin rings were observed in the absence of GFP positive surround, while GFP positive figures were also seen to surround myelin rings lacking in DiI positivity.

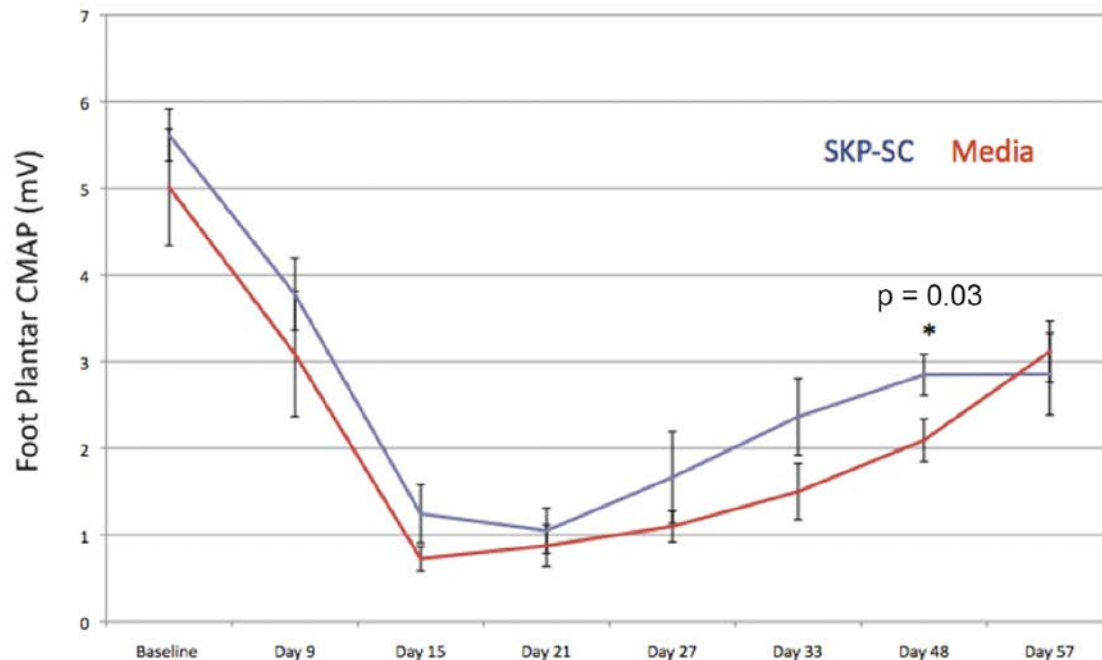


Figure 4-2 Electrophysiological recovery of tibial nerve doxorubicin injury after SKP-SC or media injection. Ten rats received bilateral tibial doxorubicin injection at Day 0; at day 9, all rats received an intrafascicular injection of 500,000 SKP-SCs to their right tibial nerve, and a media injection to their left tibial nerve (both at the site of initial doxorubicin injection). Sciatic notch stimulation CMAPs were followed for 57 days, and demonstrated an overall promotion of CMAP amplitude recovery in the SKP-SC injection group (blue tracing), that reached significance at day 48 (paired t-test, $p = 0.03$). Error bars are $\pm$ SEM.

As the GFP is expressed in the cytoplasm of these cells, lack of a GFP positive ring may represent figures at the periphery of the internode, where the cytoplasmic layer is thin.

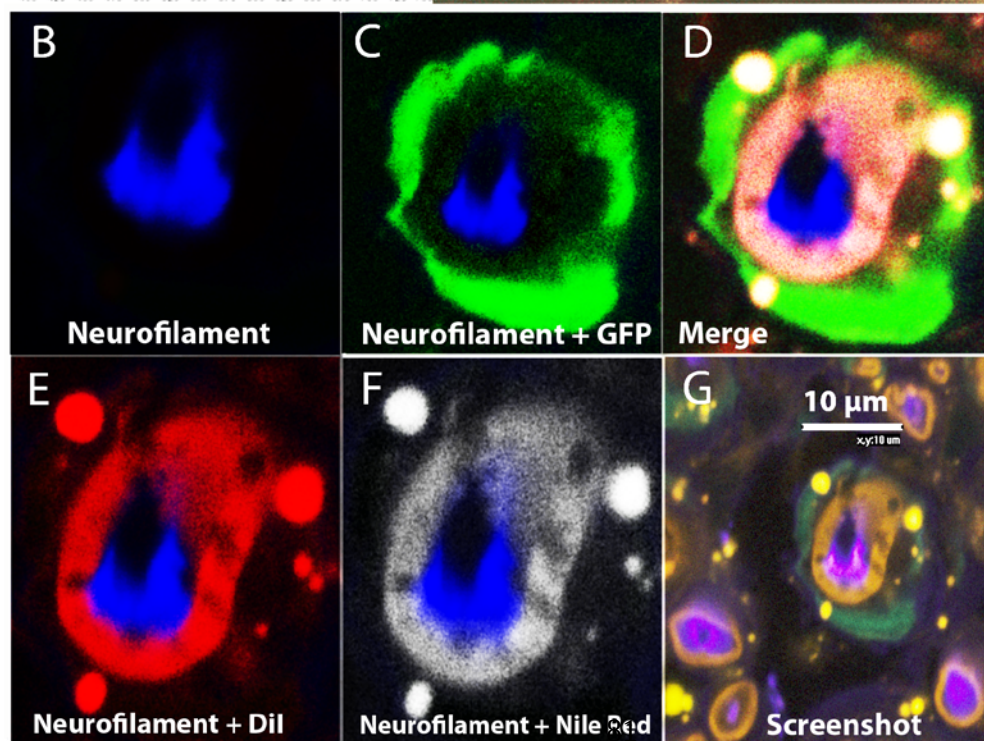
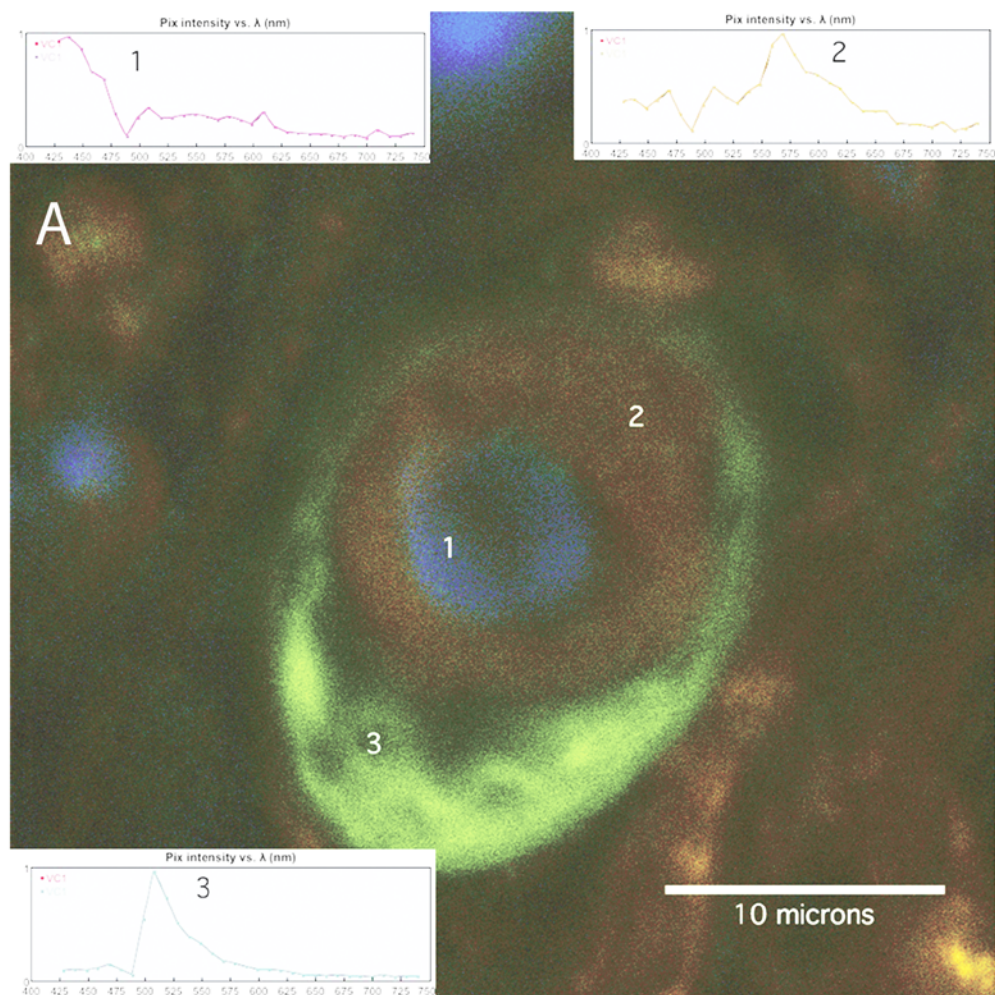


Figure 4-3 SKP-SCs myelinate tibial nerve axons after demyelination injury. A) After labeling only for neurofilament (Alexa 405 secondary), high magnification spectral confocal imaging demonstrates a single axon with SKP-SC derived myelin at day 33 post-doxorubicin (day 24 post-SKP-SC) injection (scale bar = 10 μ m). Spectral graphs of normalized emission intensity vs. wavelength confirm the presence of three distinct fluorophores (from image regions 1-3, represented by spectral graphs 1-3 respectively). GFP expressing SKP-SCs, co-labeled with DiI pre-injection, show cytoplasmic GFP positivity (3), while the myelin demonstrates DiI positivity (2), surrounding a neurofilament positive axon (1). B-F) Spectral unmix of a Nile Red stained nerve G), demonstrating co-localization of SKP-SC label DiI with myelin dye Nile Red within the compact myelin, and also within spherical cytoplasmic compartments. (scale bar = 10 μ m).

Likewise, it is possible that DiI may not exist in all cross sections of compact myelin, as it is a diffusible dye and may sequester to myelin regions outside of the imaging focus for figures with GFP positive cytoplasm. Of interest, there was very little DiI or GFP positivity to be seen in the endoneurium, outside of the context of these myelinating figures. This suggests that injected SKP-SCs that remain in the nerve at day 33 have become a part of the nerve structure. Finally, teased nerve fibre analysis demonstrated DiI positive SKP-SC myelin participating in the formation of NaV1.6 positive nodes of Ranvier (Figure 4-4), with NaV1.6 being the predominant voltage gated sodium channel type expressed at mature nodes of Ranvier in the PNS²⁵.

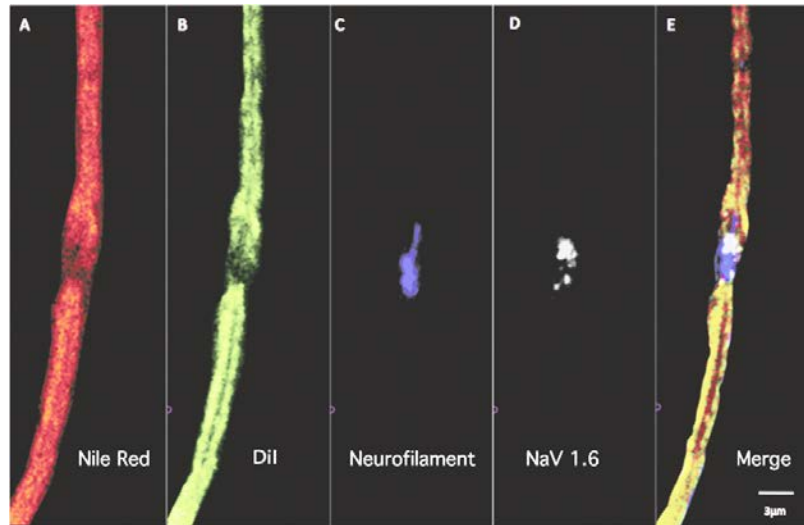


Figure 4-4 SKP-SCs participate in NaV 1.6 positive Node of Ranvier formation. A-E) Spectral unmix of a single teased nerve fibre, demonstrating SKP-SCs participating in the formation of a Node of Ranvier. A) Nile Red in compact myelin B) DiI (SKP-SC label) C) Neurofilament D) NaV 1.6 E) Merge. Scale bar = 3µm.

4.4.4 SKP-SC therapy promotes faster myelin morphometric recovery after doxorubicin injury.

EPON morphometry of the histology-group at day 33 revealed that the SKP-SC injection cohort displayed an overall tibial nerve G-ratio (0.614 ± 0.014) that was significantly lower than the media injection group (0.659 ± 0.007) (Figure 4-5) (Kruskal-Wallis test, Dunn's post test, $p < 0.05$).

The SKP-SC group G-ratio approximated the calculated ideal G-ratio of 0.60^{135} . In contrast, at Day 57 both SKP-SC and media injection groups demonstrated a G-ratio that was similar (SKP-SC 0.591 ± 0.012 ; Media 0.579 ± 0.006 , figure 5c), and likewise close to control values.

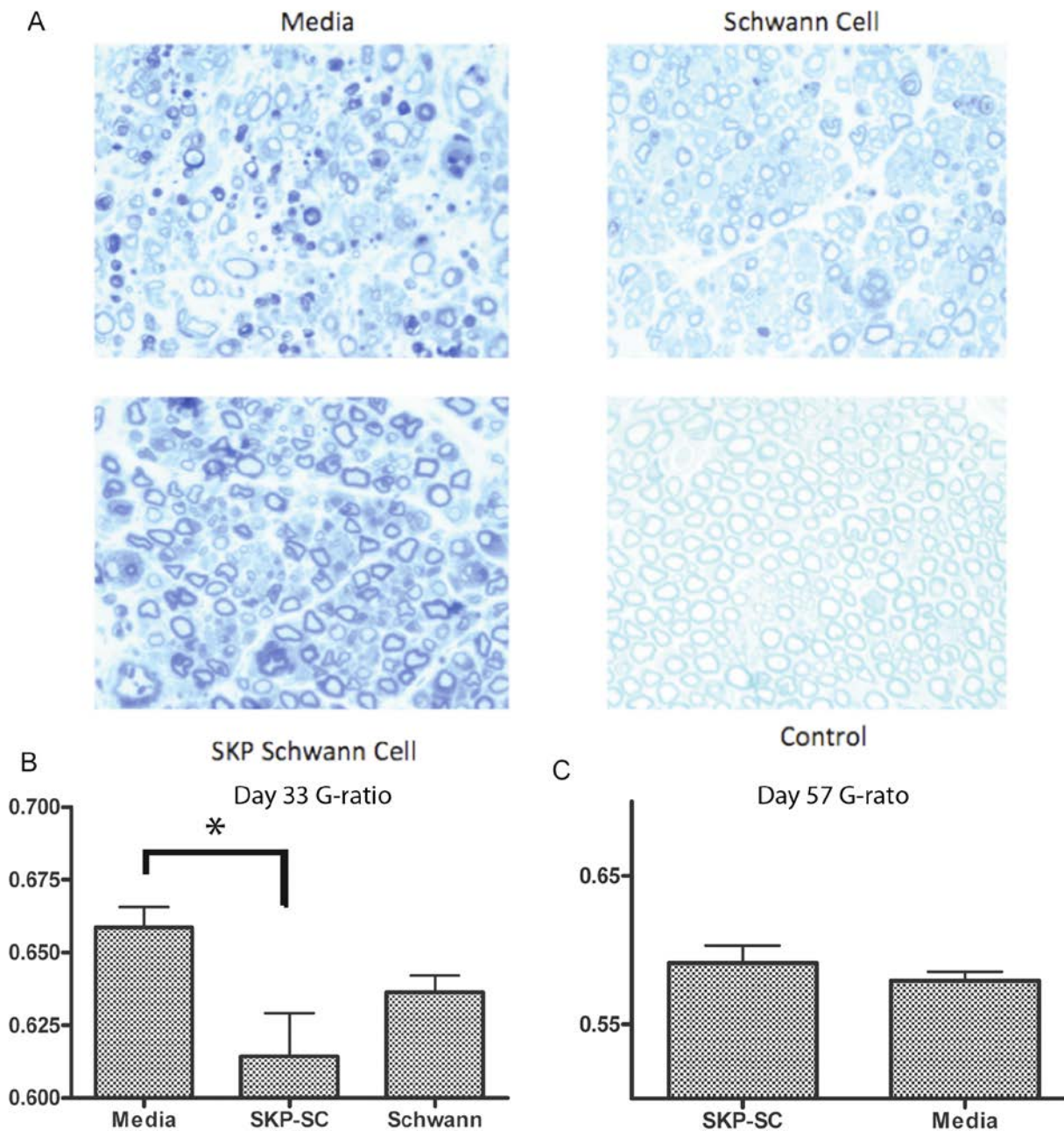


Figure 4-5 SKP-SC therapy improves early tibial nerve morphometric recovery. Day 9 SKP-SC treated tibial nerves displayed improved morphometric recovery at Day 33 post doxorubicin injection. A) 400x magnification gross morphometry of media, Schwann cell, and SKP-SC injected groups. B) SKP-SC injected nerves demonstrate a significantly lower G-ratio

than media injected nerves at this time point, while Schwann cell injected nerves do not ($p < 0.05$, Kruskal-Wallis test, Dunn's post-test, error bars $\pm$ SEM). C) At Day 57 post doxorubicin injection, day 9 SKP-SC treated nerves have a similar G-ratio (0.591) to day 9 media injected nerves (0.579), in agreement with the observed electrophysiological recovery of both groups at this time point (error bars $\pm$ SEM, student's t-test).

Our ability to determine SKP-SC derived myelin from endogenous myelin enabled the quantified comparison of SKP-SC derived (labeled) vs. endogenous (non-labeled) myelin at day 33. Of myelinated axons in three samples at day 33, SKP-SC derived myelin accounted for $15.3 \pm 5.3\%$ of myelinated figures (Figure 4-6). The "G-ratio", calculated from the immunofluorescent cross sections, for SKP-SC derived myelin (0.580 ± 0.03) was similar to that displayed by endogenous, non-labeled myelin (0.590 ± 0.02).

4.5 Discussion

Intrafascicular injection of $0.38\mu\text{g}$ ($30\mu\text{L} \times 12.5\mu\text{g/mL}$) doxorubicin into the proximal tibial nerve of the rat produces a focal conduction block that begins at approximately day 9. The conduction block manifests as reduced CMAP amplitudes and waveform abnormalities, as measured across the lesion. These results are similar to those previously described by England et al.³⁷. Distal conduction is preserved, with no significant axonal loss demonstrated in our model. This is in contrast to the findings of England et al.³⁷ who did observe some axonal loss on histological examination (although their results were not quantified). While the decrease is not significant, the average CMAP amplitude during ankle stimulation does drop to mimic the nadir of sciatic-notch derived stimulation at later time points (Day 22/29).

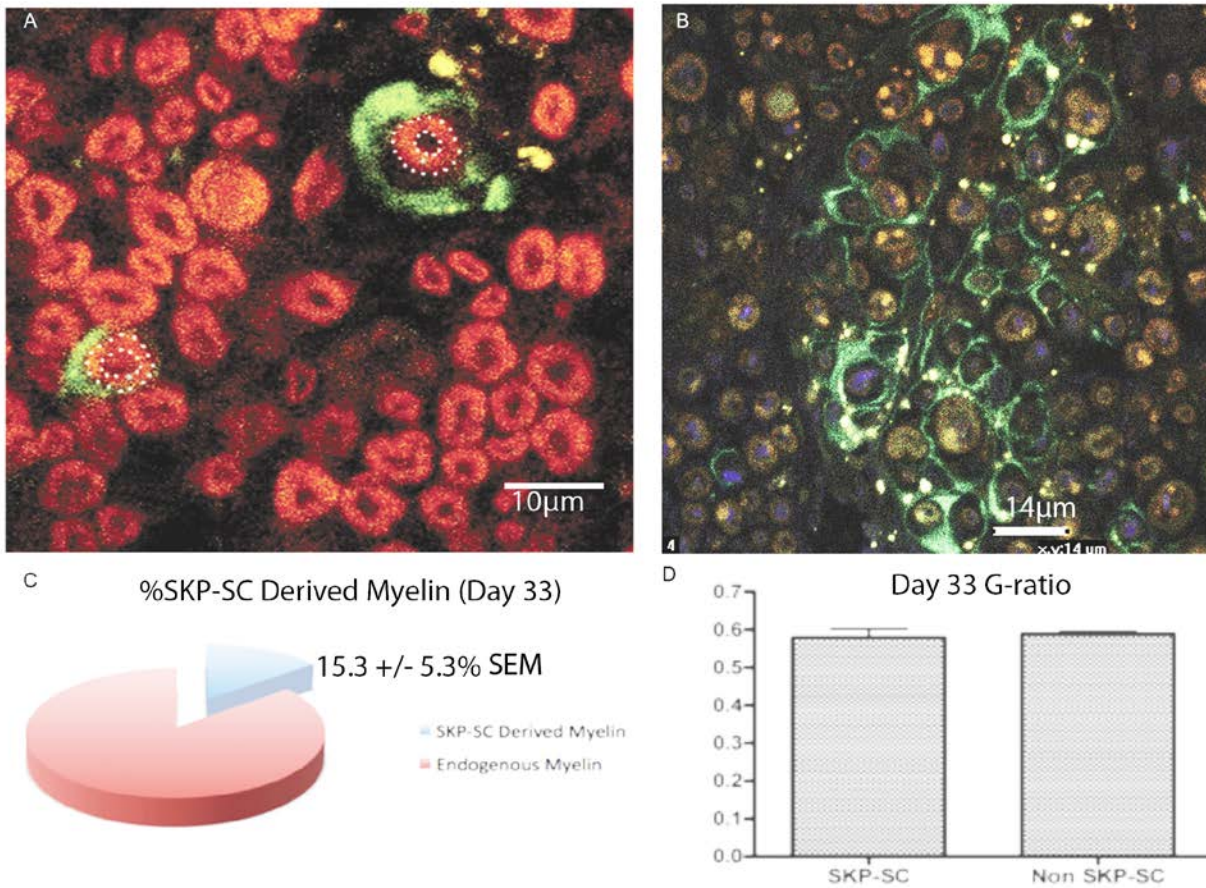


Figure 4-6 Quantification of SKP-SCs derived myelin. SKP-SC derived myelin was analyzed at day 33 post-doxorubicin injection (day 24 post cell injection). A) High magnification image (80 x 80 μ m), demonstrating myelin rings (red = Nile blue), with two SKP-SC derived myelin figures (white broken outlines). B) High magnification image (100 x 100 μ m), centered on a foci of GFP positive SKP-SCs, demonstrating robust myelination by SKP-SCs (orange = Nile Red myelin rings, blue = Neurofilament). C) SKP-SC myelin accounts for 15.3 +/- 5.3 % (SEM) of total myelin counted at day 33 (min 6%, max 24%, n=3 nerve samples). D) SKP-SC derived myelin demonstrates a similar g-ratio (0.580 +/- 0.026 SEM) to that of endogenous Schwann cell derived myelin (0.590 +/- 0.024 SEM), as calculated from similar high magnification fluorescent images.

Potentially this may reflect axonal loss that is insensitive to our quantification, or more likely anatomical diffusion of doxorubicin to also affect the tibial nerve at the ankle, albeit at a lower exposure.

To date, several types of progenitor stem cells have been suggested to participate in the formation of myelin-like figures, in both CNS^{20,115} and PNS¹⁰³, and also *in-vitro*¹⁷⁶. Of note, many *in-vivo* studies have demonstrated stem cell derived myelination through the use of graft cell injection into myelin-protein deficient mutants, allowing identification of graft derived myelin figures by observation of wild-type myelin protein^{20,103,115}. Our study conclusively demonstrates the ability of progenitor cells (SKP-SCs) to provide morphologically mature myelin in the context of competition from a normal endogenous host Schwann cell remyelination response. In particular, our data provide valuable visual evidence for the confirmation of SKP-SC derived myelin, while characterizing the morphological phenotype of SKP-SC derived myelin *in-vivo*. Moreover, we were able to quantitatively assess the extent of myelination conferred by the precursor cell therapy.

DiI served to label primarily the plasma membrane component of the SKP-SCs¹²⁴, which was seen to be incorporated into the myelin rings of Day 33 labeled figures, as well as within intracytoplasmic inclusions that were also Nile red/blue positive. The figures are in keeping currently held views regarding the formation of compact peripheral nerve myelin⁵⁰, with the inclusions possibly representing cytoplasmic lipid rich lysosomes²⁷. As GFP positive rings were potentially only seen in the central internode, and as DiI was not always seen in the myelin of GFP positive

figures, we may be underestimating the amount of SKP-SC derived myelin. Of note, performing morphometric analysis on the axial frozen sections slightly under-estimated the G-ratio, as compared to the results obtained by gold standard EPON analysis. It may be that the thickness of fluorescently labeled myelin is overestimated, in that we used the circumference of fluorescent signal to delineate the inner and outer limits of the myelin rings; it is possible that this may not represent the true border of the myelin in as accurate a fashion as semi-thin EPON morphometry. SKP-SC cell therapy was able to provide mild improvement upon the electrophysiological recovery of this segmental demyelination model. This suggests that the myelin formed by the SKP-SC is physiologic, and able to support conduction of electrical impulses. The benefit, however, was modest, and only reached significance at a single time point. In addition, the actual number of myelin figures observed, as attributable to SKP-SCs, was the minority of myelin figures at the Day 33 time point. Of note, the myelin conferred by the SKP-SCs was of a comparable G-ratio to that of the endogenous myelin at day 33, which were both close to the theoretical ideal G-ratio of 0.6¹³⁵. The results suggest that transient CMAP benefit conferred by SKP-SCs myelination may be in addition to an already robust endogenous remyelination response. In support of this notion, the CMAP cohorts demonstrated no significant electrophysiological or morphological differences at study termination, with the morphology of both cohorts approximating that of normal tibial nerve. Of note, the original research by England et al.³⁷ does demonstrate that this injury model achieves a near-full recovery by approximately post-injury day 66.

SKP-SCs may be also exerting their effects by improving the vigour of endogenous remyelination. One possibility is that the SKP-SCs are producing diffusible factors that

stimulate remyelination by endogenous Schwann cells. SKP-SCs, when transplanted into the contused spinal cord, resulted in a significant increase in the host endogenous Schwann cell response.¹¹. Furthermore, SKP-SCs have demonstrated the capability to produce neurotrophic molecules in a robust manner in vitro¹⁶¹; it is very possible that SKP-SCs may provide similar trophic support in regards to the demyelinated environment. Finally, it is possible that SKP-SCs are improving the environment for remyelination by speeding the clearance of demyelinated debris in the initial phases of doxorubicin injury. Any of the above-mentioned possibilities may also explain why the SKP-SC cohort morphometry appeared improved over even the Schwann cell cohort, and are interesting avenues of potential future research. As the above findings make SKP-SCs a promising treatment for nerve injury, these cells may also find utility in currently advancing fields of study, including peripheral nerve tissue engineering and cell-seeded artificial graft nerve-repair technology.

4.6 Conclusions

In conclusion, SKP-SC transplantation improves the recovery kinetics of the doxorubicin model of focal demyelination. The improvement is foremost from a morphometric perspective, which is also reflected in an electrophysiologic improvement, albeit to a lesser degree. Though the benefit is modest, the results are significant in considering that the therapy improved upon an inherent recovery that was already robust. On their own, these cells formed compact myelin, and participated in the formation of mature Nodes of Ranvier. Considering that these cells formed the minority of myelin figures when the morphological benefit was most pronounced, it is apparent that SKP-SCs may also be influencing the endogenous remyelination response; the mechanism by which this may occur is an interesting direction for future research.

Moreover, doxorubicin injury is an effective model system to facilitate the study of myelination by transplanted cells. The injury kinetics provide a large window of time for cell grafts to interact with the host environment, in the absence of endogenous Schwann cell competition. Furthermore, myelin derived from these cell grafts is traceable using fluorescent confocal microscopy, by localizing myelin figures associated with cellular labels such as eGFP.

Chapter Five: **High resolution spectral confocal microscopy for imaging the intact, living peripheral nervous system.**

5.1 Abstract

To demonstrate the utility and applicability of Nile Red spectral analysis to the peripheral nervous system of a living animal, we designed a novel technique for high definition, intravital spectral imaging for the tibial nerve of a rodent. Through multiple iterations, we arrived at a “nerve window” technique that has proved reliable, and free from significant motion artefact. Combined with the use of 10 μ M Nile Red applied topically to the exposed endoneurium of Thy1-GFP Sprague-Dawley rodents, we find this technique ideal for the intra-vital, spectral study of myelin through the analysis timeframe (Day 21-40) of our doxorubicin injury model.

5.2 Introduction

The advantages of intravital imaging are multifold, and have been previously summarized. In short, in vivo imaging allows dynamic assessment of neurological disease, with the direct ability to establish causal relationships between observed events¹⁰⁷. Alterations in native biological conditions resulting from the often extensive histological methods of tissue preparation can be avoided by intravital imaging. Recently, the use of “imaging windows” and “imaging chambers” has enabled the efficient sequential imaging of nervous system tissue^{35,39,41}. This is especially well established for spinal cord injury models, whereby stable repeat imaging has been established for up to 350 days after injury⁴¹. This technology has enabled increased experimental throughput and expanded the experimental indications for imaging the living nervous system in an intact animal, also known as intravital imaging.

We describe a method to obtain micrometer resolution images of nerve by combining peripheral nerve microsurgery, a tibial “window” technique, and external fixation to provide a stable platform for reliable acquisition of high-definition, 32-channel spectral data, with minimal motion artefact.

5.3 Methods

5.3.1 Animals

We used adult male Sprague-Dawley Thy-1 GFP rats¹¹⁰ (Courtesy of the Borschel lab), to facilitate intravital imaging of axons. Animal weights were all approximately 200g at the time of initial doxorubicin injury. All rats were housed in the University of Calgary animal treatment centre, and treated in accordance with all applicable animal care guidelines. All animal interventions were approved by the University of Calgary Animal Care Committee (ethics protocol # M08124).

5.3.2 Dye preparation

Nile Red dye (Sigma, USA) was prepared from powder into 10mM stock solutions in DMSO. Immediately prior to use, experimental concentrations of 10 μ M were prepared by dilution in sterile normal saline.

5.3.3 Doxorubicin injury

We have described in detail the method of tibial nerve doxorubicin injury elsewhere⁶⁰ (Chapter 4). Briefly, following a gluteal splitting exposure, 30 μ L of 12.5 μ g/ml doxorubicin (total dose of

0.38 μ g) in 0.9% NaCl (Braun Medical, Irvine CA) was injected via a 30gauge Hamilton syringe into the tibial nerve immediately distal to the sciatic bifurcation.

5.3.4 Surgical Method

Preparation: Anaesthesia was induced and maintained with inhaled isofluorane. Rats were positioned in left lateral decubitus. Medical tape (3M, Canada) was used to secure the rodent upper limbs to the anaesthesia coupler (Figure 5-1). Tape was also used to form a “corset” around the abdominal compartment of the rat (Figure 5-1), reducing abdominal breathing artefact. Skin was shaved and prepared with 70% ETOH and 10% povidine solution (Betadine, USA) from the spine to the foot.

Surgery: Before surgery, the following items were prepared: 1) 3D-printed stage, trough length 1cm, 2mm internal trough diameter 2) Silicone hemi-tube; 1cm length, 2mm internal diameter 3) Glass coverslip; 0.22mm thickness, 4mm x 10mm, fashioned with a diamond-tipped pen.

The sciatic nerve was approached using an extended gluteal splitting approach, with the overall goal of mobilizing the longest possible segment of nerve. Proximally, the sciatic nerve was exposed to the level of the sciatic notch. Distally, the approach included the exposure of the distal sciatic branches in the popliteal fossa, taking care to not injure the popliteal artery and vein. Under operating microscope magnification, external neurolysis was then performed along the entire nerve to free the tibial, peroneal, and sural divisions from each other.

Care was taken to then elevate the nerve in a tension free manner while placing the stage underneath.

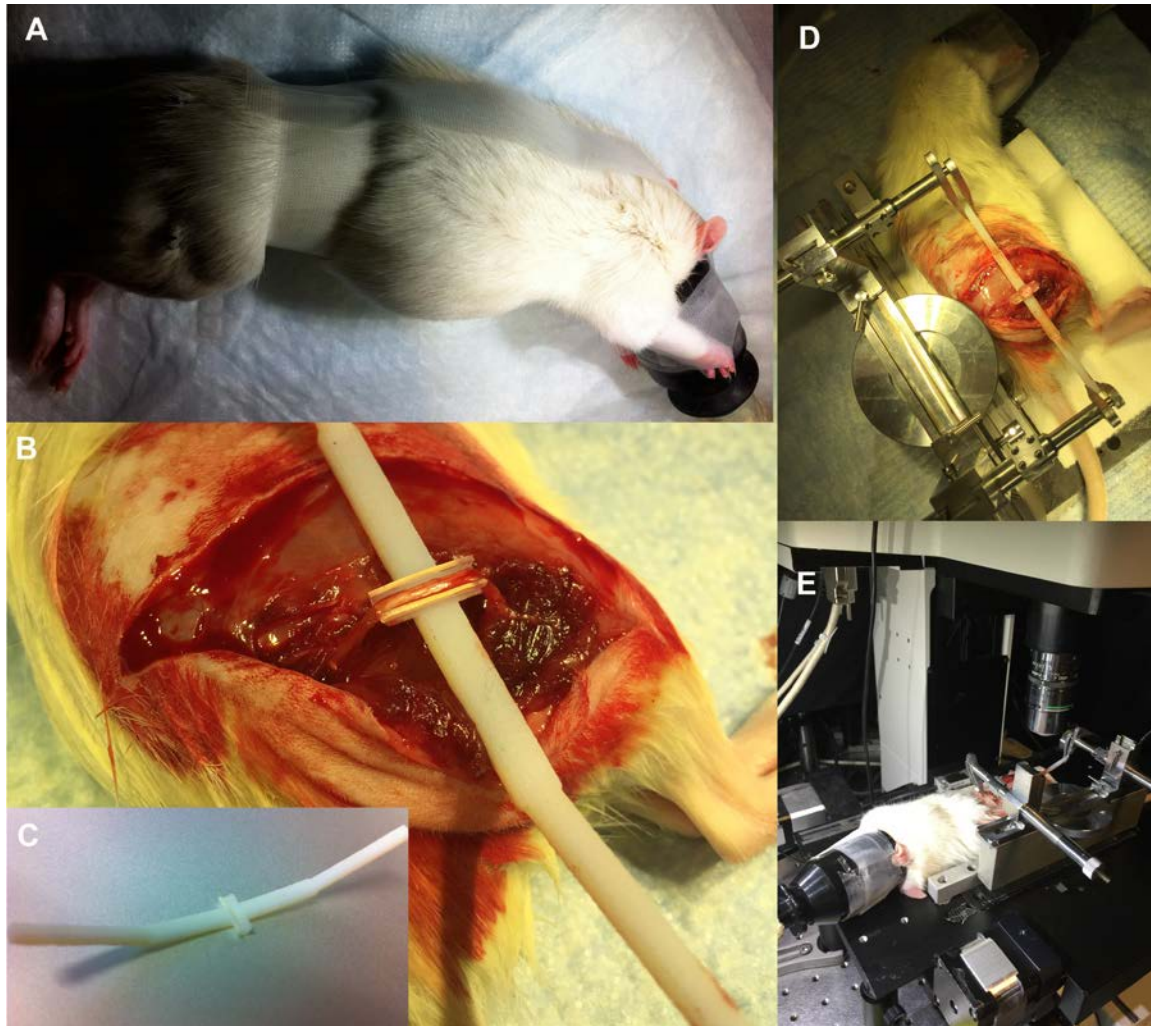


Figure 5-1 Positioning of rodents for intra-vital nerve window imaging. A) Left lateral decubitus/waist tape corset/anaesthetic mask B) Tibial nerve elevation over 3D-printed stage (C). D) Printed stage and rodent secured to the combined external fixation microscope stage. E) Combined preparation with modified Nikon A1 dropped stage.

Stage trough alignment approximated the midpoint of the sciatic exposure, which in our Thy-1 Sprague-Daley rats included the very proximal limit of the sciatic trifurcation. We then lay the nerve in the silicone hemi-tube, cradled by the nerve trough (Figure 5-2a).

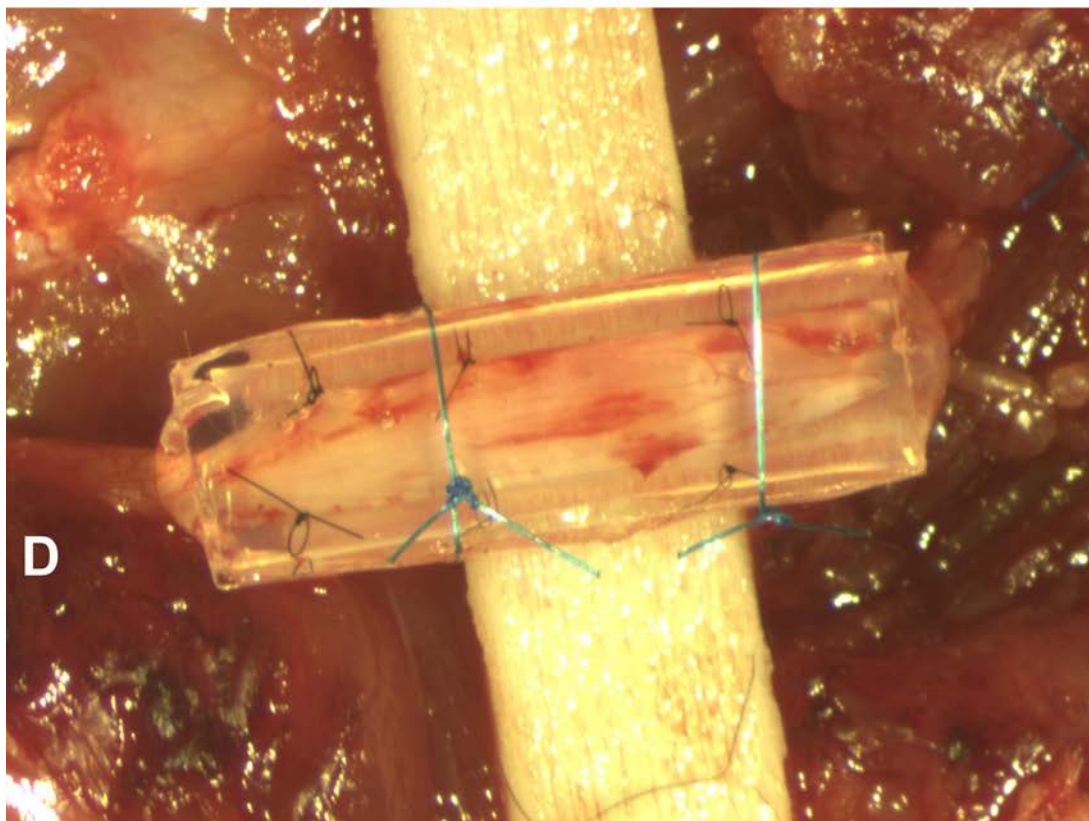
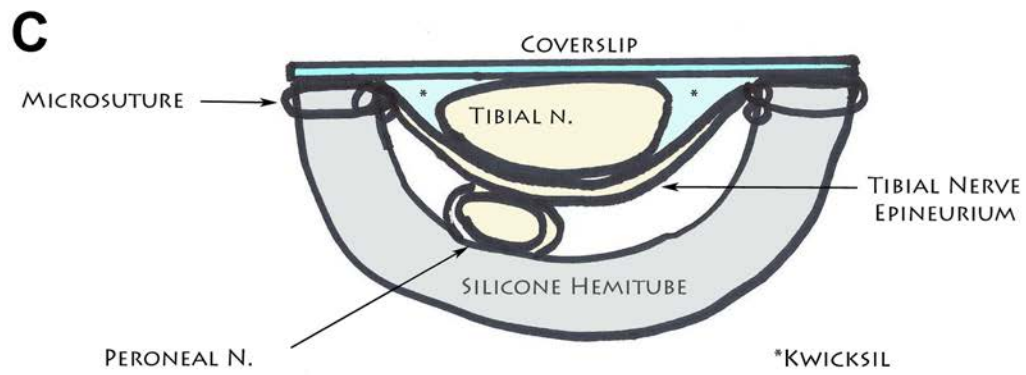
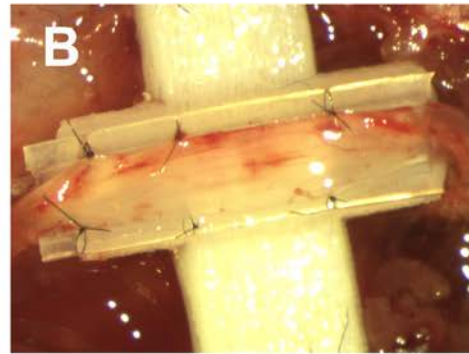
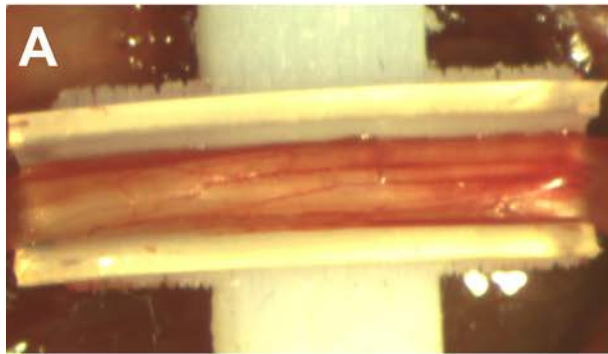


Figure 5-2 Tibial nerve window technique. A) The tibial and peroneal nerves are dissected and placed within a hemisilicone tube, in turn positioned within the groove of the 3D printed stage. B) Epineurium is opened longitudinally and tacked to the hemitube with 10-0 monofilament stitch C) A Kwiksil layer is applied superficial to the tibial nerve, followed by immediate placement of the coverslip glass. D) The final construct is secured to the stage with 7-0 prolene stitches, securing the layers during drying of the polymer.

Common epineurium was incised to mobilize the tibial nerve 5mm proximal to the tube, in order to facilitate a torsion-free construct. Tibial epineurium was then incised lengthwise, 1mm beyond the hemi-tube construct in either direction. 10-nylon sutures were used to tack the tibial epineurium to the edges of the hemitube, suspending the nerve and exposing the internal structures; the peroneal nerve thereby was placed deep to the tibial nerve within the hemi-tube (Figure 5-2c).

After achieving perfect hemostasis and a dry intraneural field, we used a fast-drying silicone polymer (Kwik-Sil, World Precision Instruments, USA) to form a thin layer between the exposed nerve and the prefabricated 0.22mm glass coverslip (Figure 5-2). To encourage intimate sealing of these layers, the coverslip, tube, and trough were secured together using 7-0 prolene, and 10 minutes was allowed to allow for hardening of the Kwik-Sil (WPI) layer.

While maintaining the lateral decubitus position, the rat was secured to the external fixator/microscope stage using medical tape, with clamp fixation of the nerve stage at either end (Figure 5-1). Then, 200 μ L of 10 μ M Nile Red in normal saline was applied superficially to the

nerve tissue by using a 30gauge needle with a 1ml syringe, to pierce the distal silicone of the nerve tube construct, and then to gently hydro dissect the superficial nerve tissue away from the silica layer using the dye solution. Special care was taken to avoid air-bubbles, while any new accumulated blood could often be “washed out” by this manoeuvre.

5.3.5 Imaging and Spectral Unmixing

All images were captured using a Nikon A1R confocal microscope on a FN1 upright frame with a modified stage base, built to allow greater clearance under the objective lens to accommodate for intravital imaging. We used a Hamamatsu 32-channel multi-anode photomultiplier array with a three-position grating turret, set to the 10nm/bin grating. The objective lens was an Apo LWD 25x Water dipping lens, NA 1.1, 2mm working distance, correction collar equipped.

After the rodent was brought to the microscope and the external fixation device set inside the “dropped” microscope stage, water interface with the objective lens was achieved using a droplet of normal saline placed on the nerve window. Images were taken in either 512 or 1024 pixel resolution with no averaging, using the 488 laser line. Spectral datasets were imported and unmixed using ImageTrak (image analysis software written by Peter K Stys <http://www.ucalgary.ca/styslab/imagetrak>, Peter Stys). The Nile Red reference spectrum was obtained by imaging fresh cut ex-vivo non-GFP labeled tibial nerve (Lewis rats), stained with 10 μ M Nile Red. The GFP reference spectrum was obtained by imaging fresh cut Thy-1 GFP tibial nerve. Approximate imaging times were 1 hour in duration, allowing for inspection of the endoneurial compartment at high magnification over a 1cm length.

5.4 Results

5.4.1 *Initial evaluations for rat sciatic intravital imaging*

Initial attempts at live sciatic imaging used no staging apparatus, along with a well of glycerol to contain the water-lens interface. Considering the long acquisition times this was highly impractical, with water leakage from the barrier being a constant issue, and movement artefact making it impossible to focus on any single area.

Our second attempt at nerve imaging involved the use of a home-made stage, to elevate the nerve from the incision and reduce the amount of breathing artefact transmitted to the nerve by alleviating it's contact with the body (Figure 5-3). Many preliminary experiments were performed using this stage. It provided adequate movement restriction, but was plagued by water-seal breakdown, especially during long imaging sessions (<10min).

Preliminary imaging sessions demonstrated the necessity of opening the epineurium to obtain adequate myelin signal (Figure 5-4). Furthermore, use of multiphoton excitation in the sciatic nerve provided no added benefit for depth of penetration (30µm max), and required the use of high laser output powers (400mW) to obtain detectable signal from the same depth as the CW laser. These 2-photon laser outputs were quickly ablative over even short imaging times. As well, we were unable to obtain 2-photon excitation spectral images when imaging through epineurium. However, to obtain adequate images past epineurium depth using the CW laser, depth of focus had to be increased to 30µm, resulting in out of focus blotchy patches (Figure 5-4C,D,E,F). In either scenario only the very most superficial plane of axons could be imaged, essentially a 30degree superficial circumference.

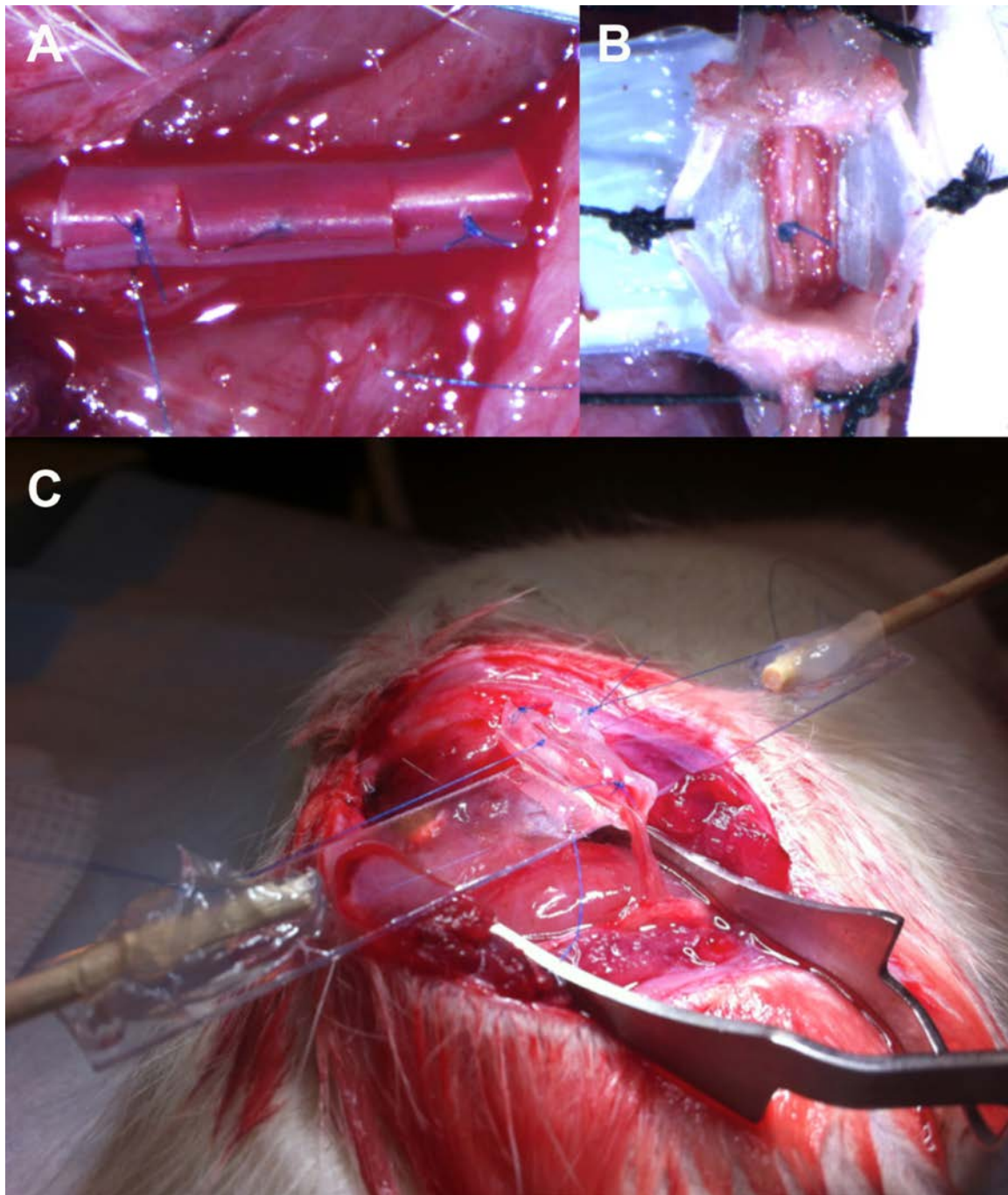


Figure 5-3 An initial attempt at a nerve stage. A plastic 1cm x 6cm stage base was fashioned from a plastic microscope slide (Fisher Scientific), and attached at either end to a wooden stick using silicone epoxy. A 1cm long plastic hemi cylindrical groove was fashioned from a

disposable pipette (Fischer Scientific), and also affixed to the stage base with silicone epoxy. A) Sciatic nerve was exposed and placed inside a 2mm internal diameter nerve tube, secured as shown. B) edges of the nerve tube were pulled open using stiches anchored to the silica, and wax inserted into the proximal and distal end of the exposure to ensure water-tightness. C) The construct was affixed to external fixation, and the stage elevated from the incision.

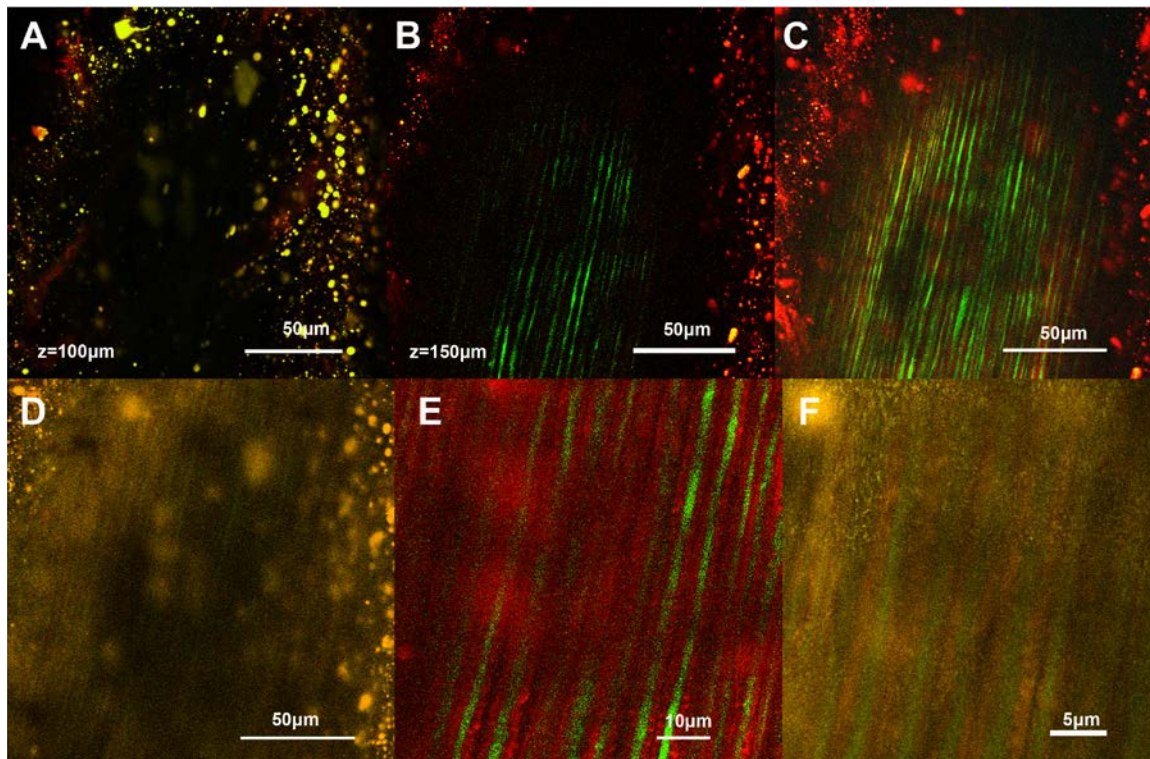


Figure 5-4 Epineurium limits the effectiveness of tibial nerve live imaging. Thy-1 GFP sciatic nerve with 10µM Nile Red. A) Sciatic epineurium, 100µm z-deep to nerve surface demonstrating extensive lipid-rich staining. B) 2-photon image 150µm z-deep, GFP axons but no myelin signal is seen. C) Same field as B) using continuous wavelength excitation (488nm), Nile Red stained myelin is visible (2-channel, 30µm depth of focus). D) Spectral capture of C. E) High magnification 2-channel zoom from C. F) Spectral high magnification zoom from D.

By combining the homemade nerve stage technique with epineurolysis we were able to obtain well-resolved images at high magnification (Figure 5-5), and over a larger area of nerve tissue. However, issues such as: 1) breathing artefact 2) water leak 3) Anaesthetic OD (to suppress artefact) were still problematic and resulted in an imaging success rate of less than 50%.

5.4.2 A tibial nerve window provides a reliable means of stable intra-vital spectral imaging.

Using a silicone tube/Kwik-Sil/coverglass (0.22mm) construct, affixed to a 3D printed stage and externally fixed to a microscope stage base, we have been able to reliably image sciatic nerve in adult rats with a high degree of success (23/25 sessions). Of the two failures, one was due to the inability to visualize axons, likely secondary to an isolated doxorubicin injury that was too severe. The second failure was technical, due to anaesthetic overdose and animal death. In all other instances, we were able to successfully recover the animal. This technique repeatedly obtains a sub-micron movement artefact (Figure 5-6), which is important for the long acquisition times required for high definition spectral imaging. Spectral images are easily resolved into their components using ImageTrak software (Figure 5-8).

5.4.3 A combination of Nile Red/Thy-1 GFP is amenable to intravital PNS morphological study

We show that 10 μ M Nile Red is an effective intra-vital myelin label, results in no appreciable deterioration of the axon/myelin morphology (figure 5-6), and reliably stains myelin with an emission spectra peak in the 600nm range (figure 5-8).

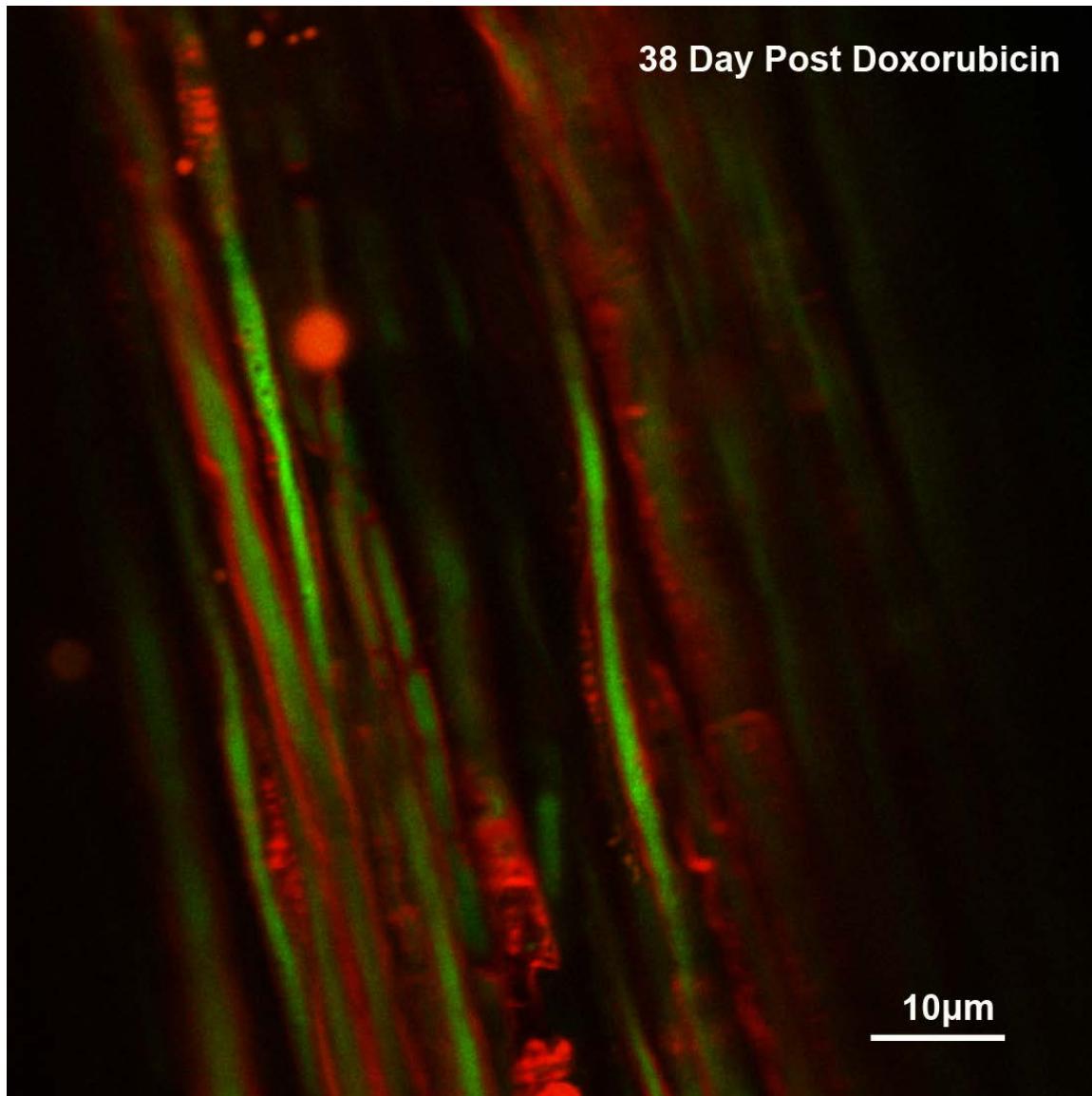


Figure 5-5 Preliminary live imaging using a suspended nerve tube construct, homemade stage apparatus, and epineurolysis. High definition 2-channel image obtained using a suspended nerve tube construct, homemade stage apparatus, and epineurolysis. Sciatic nerve is visualized 38 days post-doxorubicin injury.

Using the combination of Nile Red in a Thy-1 GFP SD rat, we qualitatively demonstrate the utility of a sciatic window technique for analysis of remyelination phenomena (Figure 5-7, 5-8). Uninjured axons retain their expected morphology (Figure 5-6). The nerve environment post-doxorubicin injury presents with increasingly thick myelin (Day 24 vs. Day 30), as well as less debris (Figure 5-7). At 40 days recovery myelin thickness has improved (consistent with prior literature⁶⁰) however the axons, in general, appear dysmyelinated (Figure 5-8). Certain myelin sheaths follow an erratic pattern, as compared to healthy myelin sheath that approximate and run parallel to the axons they surround.

5.5 Discussion

Multiple modalities are currently available for micro-imaging of the peripheral nervous system in a living organism. MRI^{8,167,168}, CARS^{6,73,76}, and Deep-OCM⁷ techniques have recently been described as effective tools in this regard. A perfect optical live imaging technology would provide rapid dynamic information at high resolution and good depth penetration, with minimal deleterious effect on biology. In reality, most current techniques are a trade-off between resolution, speed of acquisition, invasiveness, and data quality.

When we consider the plethora of genetic and intravital fluorescent labeling techniques available to interrogate nerve biology, spectral analysis is a means of making sense of potentially complicated fluorescence signals. It allows the capture of 32-channel information regarding the absolute pixel-specific intensities over the visual range of light emission, and facilitates the unmixing of multiple (4+) fluorescent signals that may be as close as 20nm apart in emission characteristics⁹². The drawback of high definition spectral acquisition is lengthy acquisition

times (up to several seconds), requiring a stable and movement-free base devoid of significant breathing or pulse artefact.

One of the major challenges to intravital peripheral nerve imaging is motion artefact, primarily due to respiratory movement that is in turn proportional to the size of the animal. CNS imaging scenarios face the same issues, but have the benefit of spine or skull bone for external fixation^{41,138}. This is an advantage that is absent for PNS imaging preparations. Many of the aforementioned studies also bypass the movement artefact problem by collecting images at video rate (10-30Hz), and discarding those images in which respiratory motion obscures the interpretation of signal. Due to current technical constraints however, video rate acquisition is restricted to only a few channels of data (usually 2 or 3). Recently, spectral imaging of the CNS has been demonstrated for six channels¹²⁹, though not at video rate. As an alternative solution, we present a tibial nerve window technique that incorporates vigorous external fixation, allowing for intravital imaging with long acquisition times. Our results indicate that the technique is capable of subcellular resolution with minimal movement artefact, allowing the collection of 32 channel spectral data.

The imaging depth achieved is only 30 μ m, meaning that this technique is limited to the analysis of a superficial layer of axons and myelin within the exposure. Despite the trialing of both 2-photon and continuous wavelength excitation, we found no benefit to multiphoton excitation for our tissue of interest.

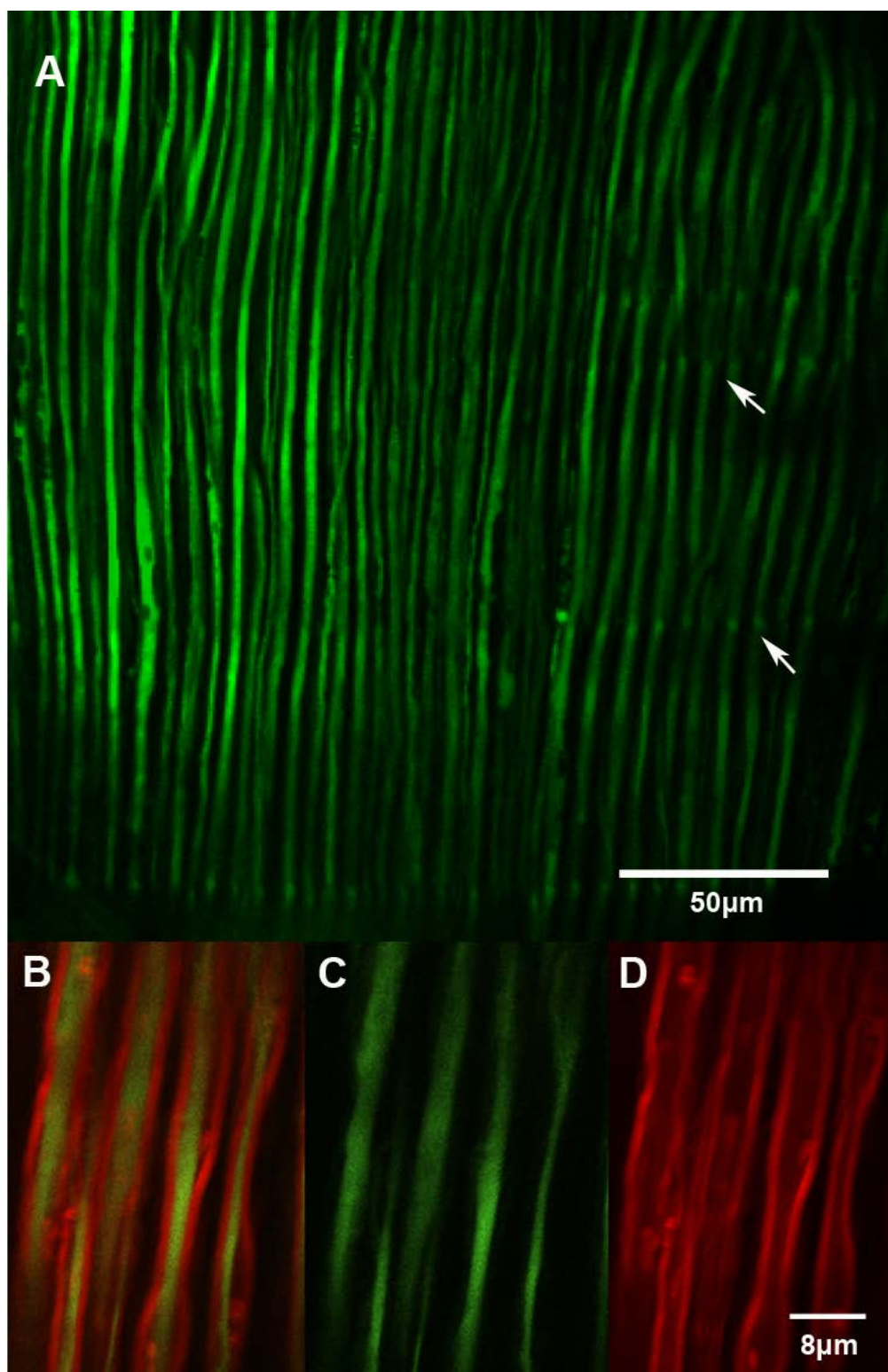


Figure 5-6 Intravital tibial nerve imaging using a nerve window. A) Wide field Thy-1 GFP axons, 3-second image acquisition time, no averaging. Movement artefact appears in respiratory periodicity (arrows), and manifests only as a small hyper-intensity in axon signal. B) Merge image, adult uninjured tibial nerve axons, high magnification C) Thy-1 GFP axons D) Nile Red stained myelin.

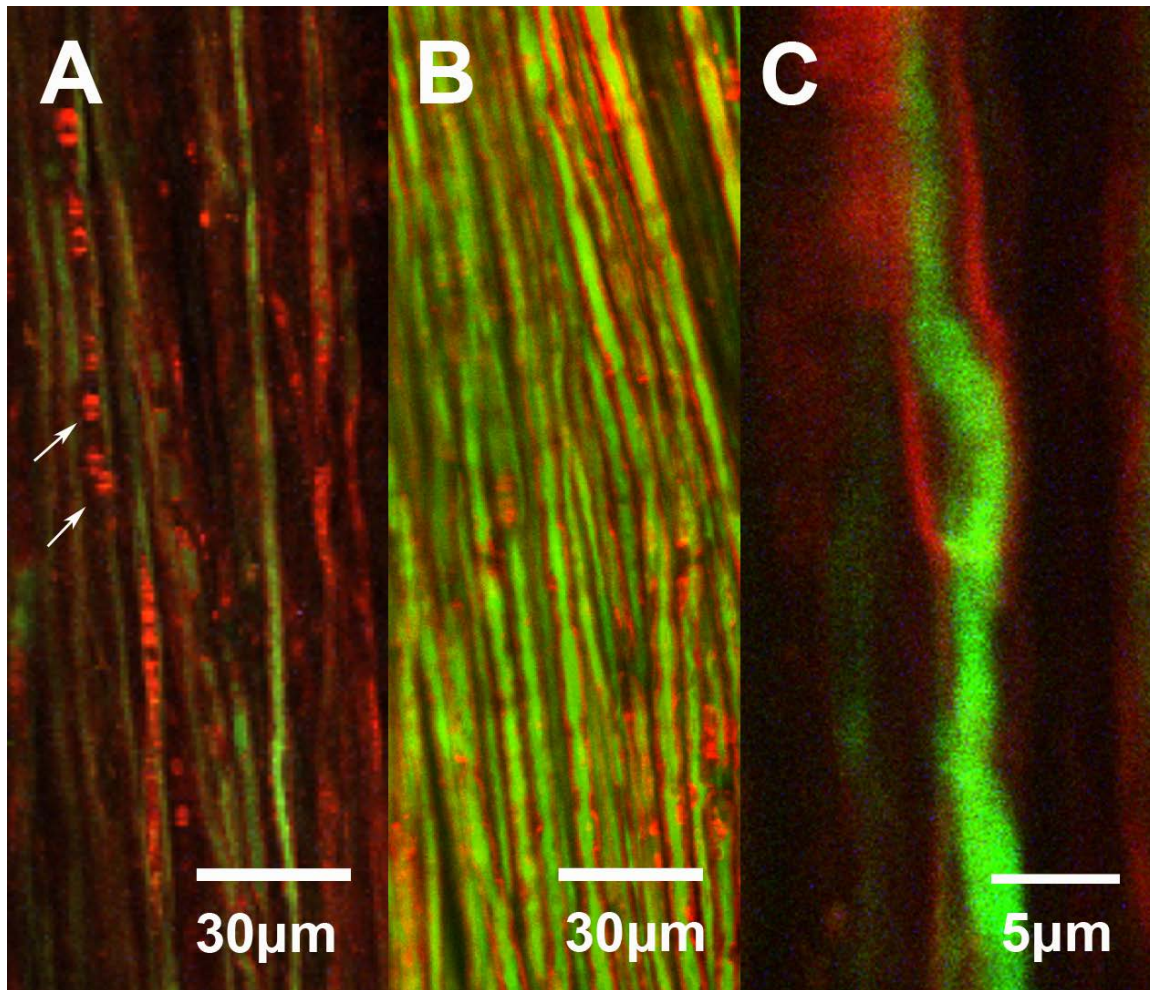


Figure 5-7 Intravital imaging of tibial nerve recovery from doxorubicin injury. A) Post-injury day 24 B) Post-injury day 30. Arrows = myelin debris. Note the spared large diameter GFP+ve axons C) High magnification zoom reveals thin, presumably new myelin surrounding an axon at day 24 post-injury, stained with Nile Red.

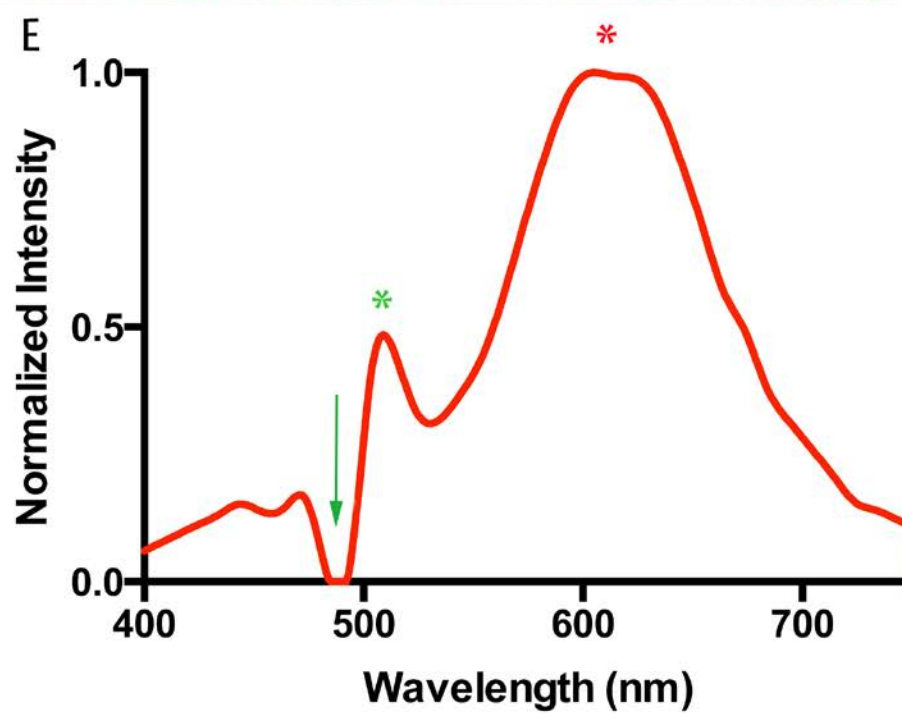
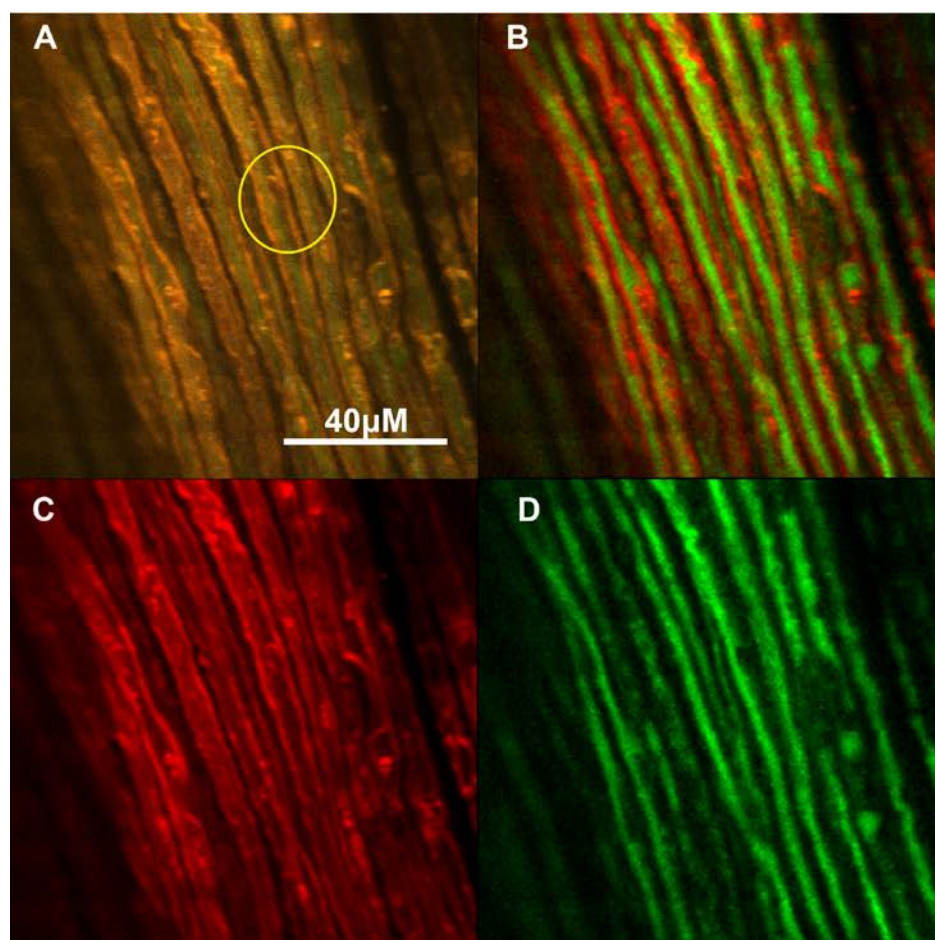


Figure 5-8 Intravital spectral imaging of doxorubicin injury. Spectral imaging and unmixing, tibial nerve post-injury Day 40. A) 32channel spectral screenshot B) Merge image of 2-channel unmix of A (GFP, Nile Red spectra) C) Nile Red D) GFP. Images reveal dysmyelination, though improved myelin thickness over previous timepoint. E) 32-channel emission spectra of the circular selection region from A, demonstrating the 488-laser finger (green arrow), the GFP emission peak (green asterisk), and the Nile Red emission peak (red asterisk).

Unlike other nervous system tissue, where imaging depths of up to a few hundred microns can be achieved⁷², imaging through myelin is particularly problematic. Studies quote a maximal 2P imaging depth of 30-50 μ m in dorsal columns³⁹, with a 2P excitation attenuation depth of 20-30 μ m due to the highly scattering properties of thick myelin⁴⁰. Our data are consistent with this.

5.6 Conclusions

A tibial nerve window is an effective method for intravital spectral imaging of a peripheral nerve environment at micrometer resolution. We have developed this technique using upright confocal microscopy and constant wavelength lasers, making the cost of the experimental setup far less than one requiring multiphoton excitation. We believe that this technique, if further adapted and improved by subsequent investigators, can have wide spread utility for the study of PNS regeneration. For our purposes, we have achieved a reliable method for investigating the tibial nerve of the rat by intravital spectral microscopy, enabling our ultimate goals of studying the solvatochromic progression of remyelination in the living rat PNS.

Chapter Six: **Spectral Analysis of Remyelination in the Peripheral Nervous System Using Nile Red.**

6.1 Abstract

Our objective for this chapter is to take our established technique of Nile Red Polarity Index Analysis, and apply it to yield useful information about the biochemistry of PNS remyelination. We have decided to follow the spectra of transplanted SKP-SC and SC derived myelin within our established doxorubicin injury model, and to compare these data across early regenerative timepoints. We go on to establish a DRG co-culture system that allows us to follow the myelin spectra of these cell types in-vitro. Finally, we revisit the doxorubicin injury paradigm and proceed to analyze the spectra of regenerating cell grafts using intravital spectral microscopy. By demonstrating a biphasic polarity index for remyelination in all in three of these designs, we provided evidence that Nile Red is capable of probing the biochemical fluctuations of early remyelination.

6.2 Introduction

Evaluation of remyelination phenomena is a difficult prospect, especially if one wishes to study myelin chemistry dynamics. To begin, axon and myelin injury are often incomplete. As healing occurs, it becomes difficult to delineate between axons that have been remyelinated and axons that were spared injury at the outset, especially if the criteria are morphometric (i.e. G-ratio). As such, most studies that have focused on the chemistry of PNS myelination have used models whereby the injury is complete and synchronous, either via axonotmetic crush injury^{56,57,125,126}, or tellurium induced focal demyelination⁶⁸.

A further difficulty is that most laboratory techniques capable of commenting on myelin chemistry lack spatial resolution or intravital applicability. For example, electrospray ionization techniques require the lipid to be purified in the aqueous phase, while gas chromatography techniques require the homogenization of the tissue sample of interest, and that it maintain its molecular structure in gaseous phase¹⁶⁴. Matrix associated desorption ionization (MALDI) does have the ability to analyze lipids in-situ with a high degree of resolution, but is limited to the analysis of thin, fresh cut tissue specimens¹⁶⁴.

We propose in this aim that the technique of solvatochromic analysis can be expanded to probe the biochemistry of remyelination at micron resolution. To this end, we have studied the spectra of early stages of myelin formation by SKP-SCs and SCs in-vitro and in-vivo, as myelin derived from transplanted cells should be one hundred percent newly synthesized myelin. Since we feel the inherent value of this technique is to probe myelin in living animals, we have included an intravital in-vivo analysis group that employs our tibial-nerve window design. *We hypothesize that the biochemical changes over the first two weeks of remyelination will result in reliable changes to the polarity index of Nile Red, and that these changes will be similar across our three separate experimental designs.*

6.3 Methods

Experimental Paradigms

6.3.1 In-vivo analysis of tibial nerve remyelination after doxorubicin injury, using fixed tissue.

6.3.1.1 Experimental design

To analyze myelin figures in-vivo after cell injection, we repeated the previous doxorubicin injury/cell injection paradigm exactly as it was described in chapter 4. The only exception was that for these new experiments, the injury and subsequent cell injections were done in a bilateral fashion. We again used Day 9 post-doxorubicin injury as the time point for cell therapy, injecting either 5×10^5 eGFP positive SKP-SCs or SCs into the injured tibial nerves of these animals.

We used 20 rats in total (10 for each cell group) with bilateral tibial nerve cell therapy, and therefore 40 separate tibial nerves injected with cells. The cell therapy of each individual rat was of the same cell type. On days of harvest, two rats (4nerves) from each cell group were collected, with the rats being chosen at random. The data from Chapter Four suggested that SKP-SC myelin morphometry at Day 33 approximates that of normal nerve; we therefore chose our harvest timepoints as post-injury Day 21, 24, 27, 30, and 33.

6.3.1.2 Tissue processing and dye staining

The methods of harvest, fixation, cyroprotection, tissue freezing, cutting, slide preparation, Nile Red staining (10 μ M), and imaging were identical to those described in Chapter 4. We used 12 μ m thick tibial nerve axial sections. Images were analyzed using the 488-laser line to enable excitation of both GFP and Nile Red.

6.3.1.3 Image analysis

32-Channel spectral images were imported into Imagetrak, and converted to spectral pseudocolor images (Figure 1c) using the established Nile Red Polarity Index method (see common methods). Myelin selection regions were chosen based on the presence of surrounding GFP positive cytoplasm, and selection regions were drawn my hand.

6.3.1.4 Statistical analysis

Analysis of curve differences was obtained by 2-way ANOVA ($\alpha 0.05$), using the Tukey's post-test for multiple comparisons where applicable. We considered one myelin selection region to constitute a single "n" for our statistical analysis. Statistics were only performed on the peak value of each individual PI curve, representing the most common peak spectral value seen in all of the pixels for that region. Curve averages were calculated by averaging the normalized magnitude of a particular PI partition across all selection regions. Therefore, the average peak PI is not necessarily the peak value of the averaged curves for these analysis.

6.3.2 *In-vitro analysis of live myelination*

6.3.2.1 Experimental design

We used a co-culture system of DRG explants that pan-express GFP, and applied either BFP+ve SCs or SKP-SCs to the co-culture system before inducing myelination via addition of ascorbic acid. We designated 3 plates per cell condition for each of the following days post-myelin induction: Day 10, Day 13, Day 16, Day 19, Day 22, Day 29, Day 35. These days were chosen to represent timepoints whereby we would likely see myelination, which is usually felt to begin in-vitro (at the earliest) 7-10 days after the addition of ascorbic acid^{148,171}. The DRG

chemotherapy regimen was modified to allow the survival of scant endogenous GFP Schwann cells, such that during co-culture these cells might also proliferate and myelinate, allowing the direct comparison of spectra from supplemented BFP positive cells against those endogenous to the dish.

6.3.2.2 Cell Culture and Viral Transduction

The methods described for harvesting, culture, and transduction of SKP-SCs and Schwann cells are described in the common methods section.

6.3.2.3 DRG Harvest/Culture

Before harvest, 3cm Flurodishes (World Precision Instruments) were coated as follows. We combined Laminin (4 μ g/ml), PDL (20 μ g/ml) (both BD Biosciences), and 1mL rat tail collagen Type 1 (Sigma) in 10mL sterile chilled water, filtered it through a 70 μ m filter, and added 500 μ L Matrigel (Corning) to the ice-cold filtrate. Dishes were exposed to this solution for 10 seconds, with the excess removed by pipette. They were left to dry for 2 hours.

Postnatal Day 3 rat pups (pan-GFP rats) were sacrificed by decapitation and placed immediately on ice. After exposing the spine a complete rostro-caudal laminectomy was performed, exposing the spinal cord and allowing access to the DRGs. DRGs were harvested with a #5 jeweller's forceps and stored in chilled Neurobasal media. We harvested 25 DRGs maximum at a single session (1 pup), taking approximately 10 minutes, in an effort to minimize the decay of the tissue. DRGs were then trimmed of excess rootlets using a dissection microscope, and plated under the culture hood using 2ml maintenance media (1 DRG/dish). DRG maintenance media

was Neurobasal media, 20% FBS , 1% Pen/Strep, 0.04% Fungizone (all Gibco), and 50ng/mL Nerve growth factor (Cedarlane). In addition, DRG media initially contained 7 μ M cytosine arabinoside, and the DRG dishes were left undisturbed in the culture incubator for 4 days. After this time, culture were inspected daily, and media changed q2days. When ample neurites and minimal endogenous Schwann cells were present, usually at day 6, the cytosine arabinoside was removed from the culture condition. DRGs were then washed 3 times over 3 sequential days with chemotoxin-free maintenance media before co-culture was performed.

6.3.2.4 Co-culture

Co-cultures were established by seeding either BFP-SKP-SCs or BFP-SCs at a density of 25,000cells/mL (2ml/dish) using established techniques⁷⁰. These co-cultures were grown for approximately 6 days in differentiation medium (3:1 DMEM/F12 (Gibco) with: NGF 1:20000, N2 supplement (Gibco) 1%, Anti-anti (Gibco) 1%, Neuregulin 1:400, Forskolin 1:5000, and 5% FBS (Gibco). After BFP positive Schwann cells were visualized aligning with axons, media was changed to myelination medium (as above with 0.1% Ascorbic acid, and without Neuregulin and Forskolin). Media changes were every 3 days.

6.3.2.5 Imaging of Live Cultures

All cultures were imaged with the Nikon C1si inverted microscope (Nikon USA) in combination with a 32channel spectral detector (Hamamatsu 32 channel multi-anode photomultiplier array, Hamamatsu Japan). On imaging days, selected co-culture dishes were given a media change consisting of maintenance media plus 10 μ M Nile Red. This was applied for 30 minutes, followed by a double rinse of maintenance media (5 minutes each). After this, cultures were

bathed in 2ml maintenance media, and immediately transferred to the imaging facility. Culture dishes were allowed to normalize their temperature and carbon-dioxide levels for 20 minutes, and stored in an adjacent incubator until imaging. Individual co-culture dishes were transferred to the microscope directly and imaged for no longer than 7 minutes before fixing with formalin, in order to maintain a euthermic imaging environment (37°C). We used a 60x oil immersion objective and the 405/488 laser lines to image culture dishes. Analysis images were obtained by using the 488 laser line only.

6.3.2.6 Image Processing

Spectral images were imported into Imagetruk and converted to quantitative pseudocolor images for PI analysis. Each image was also unmixed into a three-channel image, using BFP, GFP, and Nile Red (measured from preliminary in-vitro specimens) as the base spectra. Myelin conferred by BFP+ve cells was usually obvious by looking at the raw spectral images, and most selection regions were delineated in this fashion. Other myelin selection regions were obtained by comparing the 3-channel spectral-unmixed images with the raw spectral image, to determine which myelin segments were being conferred by BFP-labeled cells. This selection mask was then applied to the quantitative pseudocolor image, and myelin segments analyzed one at a time for PI.

6.3.2.7 Immunohistochemistry

In-vitro immunohistochemistry - Immediately after imaging, culture dishes were rinsed twice with PBS and fixed with 10% Formalin for 20 minutes. The dishes were stored in dPBS with 0.1% sodium azide, away from light. For primary antibody staining we rinsed twice with PBS

for 5 minutes, then applied 0.1% Triton in PBS for 5 minutes. We again rinsed in PBS for 5 minutes before applying 5% bovine serum albumin (Sigma) in PBS for 1hr. The BSA solution was removed and primary antibody solution applied overnight at 4°C. For secondary antibody staining we rinsed the cultures twice with PBS (5min), and applied (1:200) secondary antibody in PBS directly for 2 hours at room temperature. Cultures were then rinsed twice with PBS (5min) before imaging. For neurites we used SMI312, 1:800 (abcam)/Alexa 488; for BFP labeled cells we used BFP, 1:200 (Biovision)/Alexa 405; for myelin basic protein we used MBP, 1:200 (Santacruz)/Alexa 555. We did not re-apply Nile Red for immunohistochemical analysis

6.3.2.8 Statistical analysis

Analysis of curve differences was obtained by 2-way ANOVA ($\alpha 0.05$), using where appropriate the Tukey's post-test for multiple comparisons where applicable. We considered one myelin selection region to constitute a single "n" for our statistical analysis. Statistics were only performed on the peak value of each individual PI curve, representing the most common peak spectral value seen in all of the pixels for that region. Curve averages were calculated by averaging the normalized magnitude of a particular PI partition across all selection regions. Therefore, the average peak PI is not necessarily the peak value of the averaged curves for these analysis.

6.3.3 Intravital analysis of tibial nerve remyelination after doxorubicin injury

6.3.3.1 Experimental Design

We again employed our established doxorubicin injury model using Thy-1 GFP rats (as per Chapter 5) and performed a unilateral right-sided tibial nerve injury at Day 0. We then administered either 5×10^5 BFP+ve SKP-SCs or BFP+ve SCs to the injury site at Day 9, for subsequent intra-vital analysis (as detailed in Chapter 5). Our analysis time-points were Day 21, 24, 27, 30, 33, and 40 post-injury. We analyzed $n=3$ rats for each timepoint, and were able to obtain a further long-term measurement from a single rat at Day 50 for the Schwann cell group.

6.3.3.2 Imaging

Intravital imaging (surgery, nerve window application, Nile Red application) proceeded as described in chapter 5, allowing us to capture 32-channel spectral data sets. We again employed an ApoLWD 25x water-dipping lens, NA1.1, with a 2mm working distance. We used a simultaneous excitation of 405nm and 488nm laser emission to capture both spectral and 3-channel images. Of note, we found the 405 laser had minimal ability to excite Nile Red at the power ranges we employed (Data not shown), and were satisfied that Nile Red spectral emission was due only to the 488 excitation.

6.3.3.3 Image processing

Spectral images were imported into Imagertrak and converted to quantitative pseudocolor images for PI analysis. Each image was also unmixed an addition time into a three-channel image, using BFP, GFP, and Nile Red (measured from control sciatic nerve in vivo) as the base spectra. Myelin selection regions were obtained by comparing the 3-channel spectral-unmixed images with the raw spectral image, to determine which myelin segments were being conferred by BFP-

labeled cells. This selection mask was then applied to the quantitative pseudocolor image, and myelin segments analyzed one at a time for PI.

6.3.3.4 Statistical analysis

Analysis of curve differences was obtained by 2-way ANOVA ($\alpha 0.05$), using where appropriate the Tukey's post-test for multiple comparisons where applicable. We considered one myelin selection region to constitute a single "n" for our statistical analysis. Statistics were only performed on the peak value of each individual PI curve, representing the most common peak spectral value seen in all of the pixels for that region. Curve averages were calculated by averaging the normalized magnitude of a particular PI partition across all selection regions. Therefore, the average peak PI is not necessarily the peak value of the averaged curves for these analysis.

6.4 Results

6.4.1 Schwann cells and SKP-SCs myelinate axons in all paradigms tested

We observed extensive Schwann cell-derived (Figure 6-11, 6-3, 6-6) and SKP-SC-derived myelin (Figures 6-4, 6-7) in all three of the experimental designs. The myelination was sufficient to allow selection regions to be outlined by hand, sometimes at a micron scale.

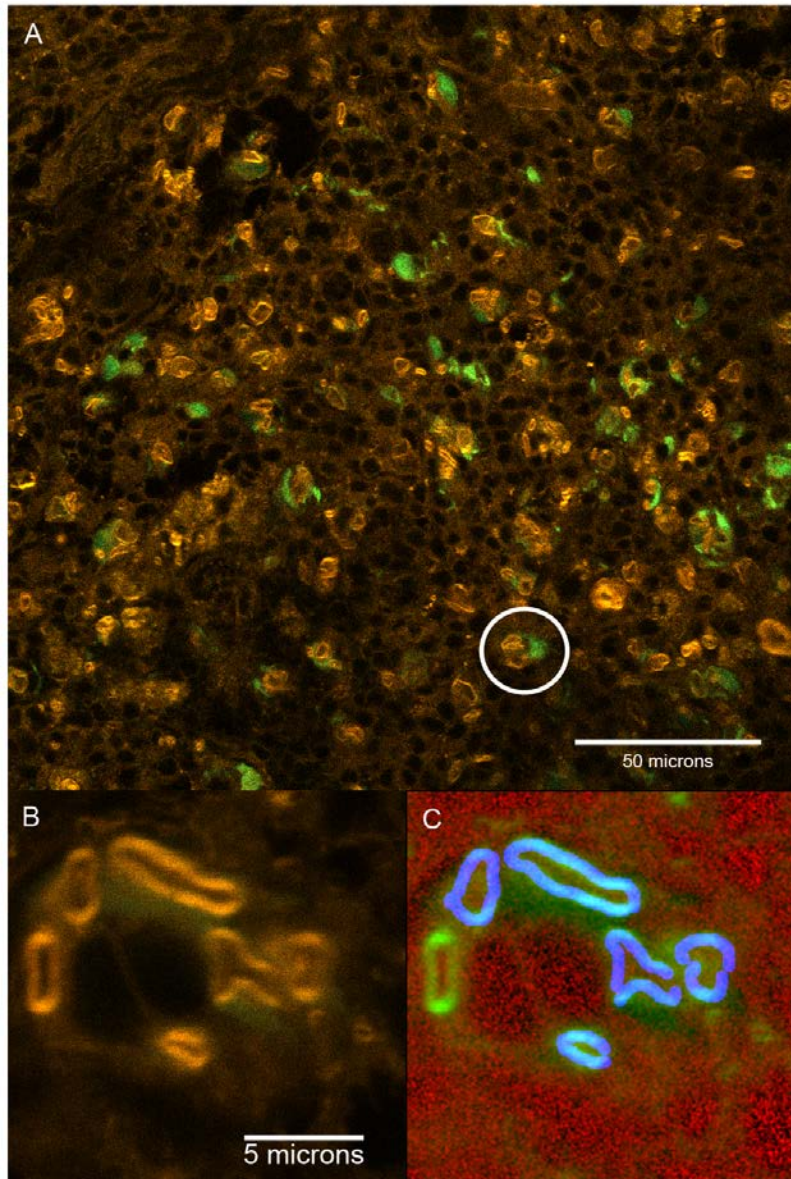


Figure 6-1 Fixed tissue analysis of SKP-SC and Schwann cell myelination. 24 days after doxorubicin injury and 15 days after cell injection, GFP positive Schwann cells provide myelin to demyelinated axons (not labeled). A) Wide field image of a 12µm thick axial section of tibial nerve. Myelinating GFP positive SCs are visualized (Myelin stained with Nile Red) B) Zoom of the circular selection region from A. C) Quantitative pseudocolor image of B, demonstrating hand-drawn blue selection regions specific to myelin conferred by GFP positive Schwann cells.

6.4.2 Regenerating myelin displays a biphasic polarity index curve.

For all paradigms analyzed we show that myelin polarity progresses through a biphasic polarity index with increasing myelin maturity.

6.4.2.1 Fixed tissue analysis

In contrast to the polarity index of developing myelin (that decreases progressively with increasing age), we demonstrate that newly regenerated myelin has a polarity index comparable to that of adult myelin.

The averaged polarity index curves represent amalgamated histogram data pertaining to the distribution of peak spectral frequencies per pixel of myelin analyzed. They therefore contain general information about the “distribution” of these peak frequencies that is apart from the “averaged most common” peak frequency, as represented in Figure 6-2C. Without being quantitative, we can observe that young myelin, while having a comparatively low PI, it also has a wider and more varied distribution of measured peak frequencies. These distributions “tighten up” after Day 27, with the elimination of secondary spectral peak populations (Schwann cell day 21, 27, Figure 6-2B), and the narrowing of the curve distribution by day 33.

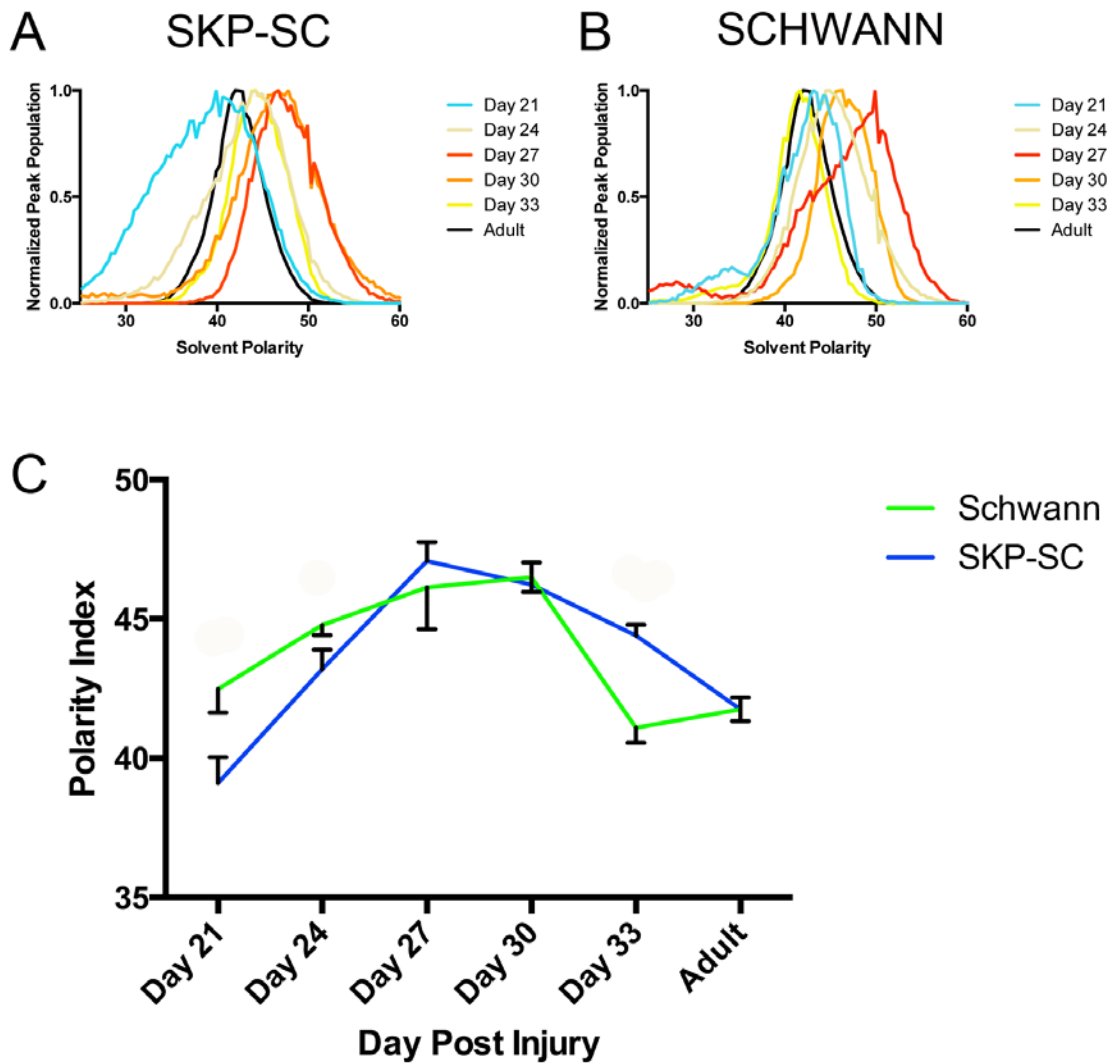


Figure 6-2 Fixed tissue polarity index analysis of SKP-SC and SC conferred myelin A)

Averaged polarity index curves from SKP-SC myelin selection regions (Day 21 n=24, Day 24 n=24, Day 27 n=24, Day 30 n= 33, Day 33 n=37, Adult n=10) Young myelin in general has a blue shifted polarity index. The polarity index of myelin increases through Day 24 and 27, before returning toward the polarity index of adult control myelin by Day 33. B) Averaged polarity index curves from Schwann cell myelin selection regions (Day 21 n= 14, Day 24 n=22,

Day 27 n=16, Day 30). n=19, Day 33 n=17, Adult n=10). A similar trend is observed with young myelin progressing from a blue shifted polarity index to a red shifted polarity index by Day 27 and 30, following a return to approximate the polarity index of adult control myelin by day 33. C) Averaged polarity index peak values for myelin selection regions as outlined in A and B. See appendix figure one for full statistical analysis.

Furthermore, there is a trend for the PI of regenerating myelin to become increasingly polar over a 6-9 day time period, and to display a maximum rightward shift at Day 27 or 30 in our model. Following this, there is a return toward the PI of adult control myelin, of which the Day 33 time-points of both cell groups begin to approximate.

Within the context of this observed trend, there are time-point specific differences apparent for the PI of SKP-SC and Schwann cell transplant-derived myelin (Day 21- SKP-SC<SC; Day 33- SKP-SC>SC; 2 way ANOVA (α 0.05), Tukey's post-test. There is not a significant difference between the two curves on the basis of cell type (2-way ANOVA α 0.05). However, the “Day Post-Injury” is a highly significant factor (2-Way ANOVA, α 0.05, $p<0.0001$), and in general demonstrates that the bluest shifted myelin (Day 21, Adult), is significantly different in PI than the most red-shifted myelin (Day 27,30). See Appendix for full 2-way ANOVA.

6.4.2.2 In vitro analysis

We compared the in-vitro spectra of myelin derived from co-cultured BFP+ve SKP-SCs and SCs, not only with each other, but also with the myelin derived from the GFP Schwann cells endogenous to the co-culture. We did this using living co-cultures. Robust myelination could be

quantified in all conditions, beginning at 13 days after the induction of myelination with ascorbic acid. Our SKP-SC group produced MBP, that was robustly apparent 16 days after induction (Figure 6-4).

Our co-culture conditions contained mixed cell types. The cultures commonly demonstrated GFP positive neurites radiating out from the central DRG, followed by GFP positive Schwann cells migrating distally. BFP-positive cells (Schwann cells and SKP-SCs) would usually plate down in a more peripheral location, and migrate inwards to associate with these neurites (Figure 5-3). Also present were endogenous fibroblasts, whose liposomes brightly stained for Nile Red¹²³. Our experiment was limited in length by the viability of DRGs in the co-culture after 22 days (a total of 40 days culture time for the DRGs). Co-cultures at Day 29 and 35 showed marked degradation of the culture conditions, and were excluded from the analysis.

The comparison of the myelin PI of these four groups yielded a similar dynamic to that observed in our fixed tissue experiment. Of note the PI of the living in-vitro myelin was markedly more polar than that of the fixed tissue myelin.

For the myelin specific PI derived from the BFP cells, there was no significant difference between the curves of these two cell conditions (2 way ANOVA, $\alpha 0.05$). Within an individual cell type, the differences in PI amongst time points was often very significant, demonstrating that we are observing a real effect with the dynamic changes in myelin spectra (2-way ANOVA, Tukey's post test, Appendix Table 2a).

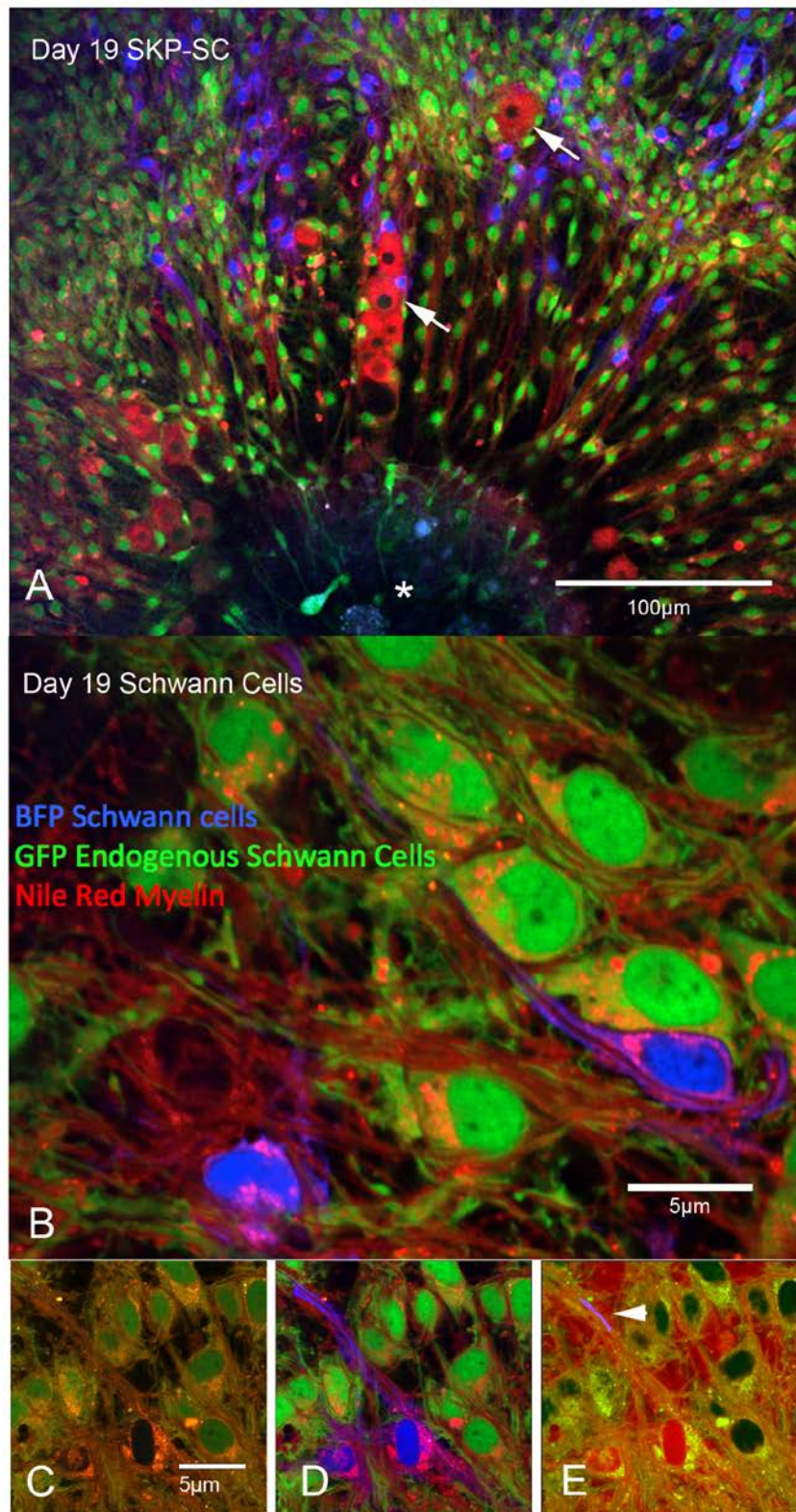


Figure 6-3 In-vitro co-culture of DRGs with SKP-SCs /Schwann cells . BFP positive cells (SKP-SCs, SCs) provide myelin in-vitro to DRG neurites, as seen in live culture images at day 19. A) Wide field 3 channel unmix image (Blue = BFP, Green = GFP, Red = Nile Red), demonstrating a mixed co-culture. BFP+ve SKP-SCs cells are seen together with endogenous Schwann cells, aligning along neurites that radiate from the DRG (*). Present also are other highly Nile Red positive cells, likely fibroblasts (arrows), consistent with previous literature¹²³. B) Zoom image of BFP (transplanted) Schwann cells and GFP (endogenous) Schwann cells clearly myelinating in culture. C-E) Method of analysis; myelin selection regions (E, blue-line/arrowhead) are determined by hand upon inspection of the screenshot (C), the 3-channel unmix (D), and transferred to the quantitative pseudocolor image (E).

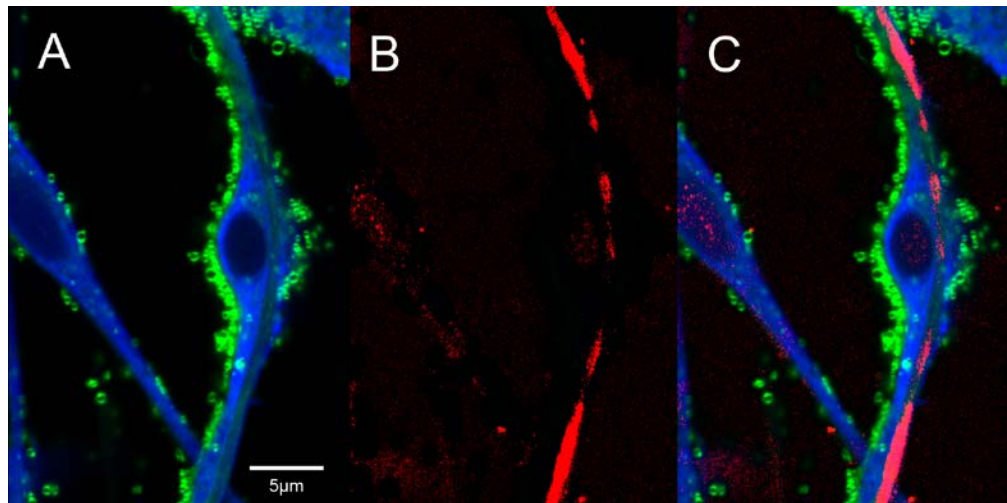


Figure 6-4 SKP-SCs provide MBP positive myelin in fixed co-culture. 16 days after myelin induction. 3-channel unmix (blue = BFP, green = Nile red, Red = MBP/Alexa555). A) Blue and Green channel. Nile Red stains the myelin space. Also much of extracellular associated Nile Red has formed into spherical figures after histologic processing. B) Red Channel, MBP C) Merge.

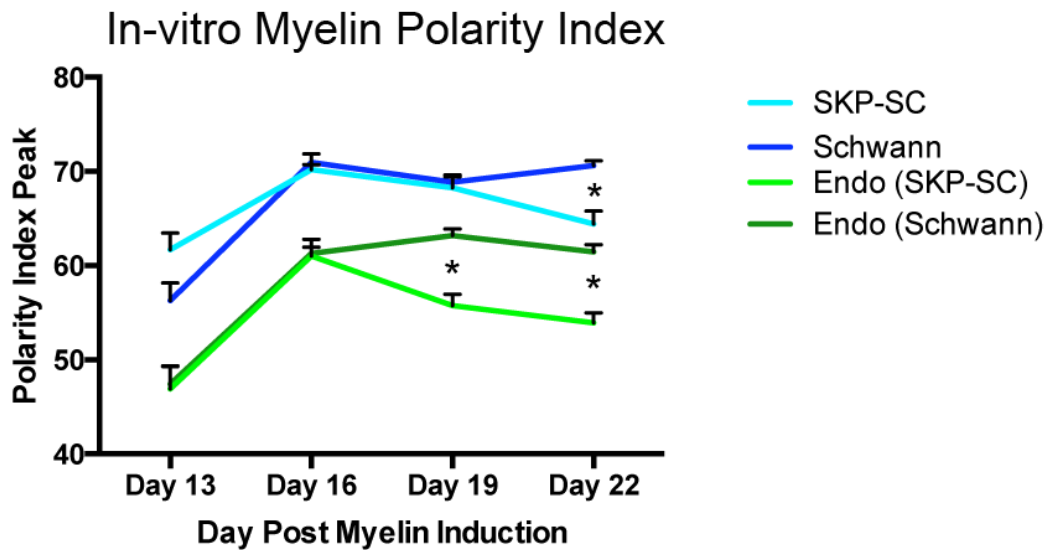


Figure 6-5 In-vitro polarity index myelin analysis. Averaged Peak Polarity index of in-vitro myelin selection regions from transplanted BFP+ve Schwann and SKP-SCs, as well as the endogenous Schwann cell populations of those respective conditions (Endo-SKP, Endo-Schwann). In general, early in-vitro myelination is characterized by a relatively low polarity index. This is followed by an abrupt increase at Day 16, and then a gradual decline with increasing age. The myelin spectra from both SKP-SCs and endogenous Schwann cells co-cultured achieve this dynamic faster than their Schwann cell co-culture counterparts (Day 19 and 22 Endo(SKP) vs. Endo(SC); Day 22 SKP-SC vs. SC; 1-way ANOVA $p < 0.05$) In addition, the PI curves of transplanted cells are significantly different from their endogenous counterparts (2-way ANOVA $\alpha 0.05$).

The only significant difference between SK-SC and SC myelin PI occurred at day 22 (2 way ANOVA $p < 0.01$). For BFP-SKP-SCs: Day 13, $n=18$; Day 16, $n=15$, Day 19, $n=15$; Day 22, $n=20$. For BFP-Schwann cells: Day 13, $n=19$; Day 16, $n=15$; day 19, $n=29$, day 22, $n=30$.

For the myelin specific PI derived from endogenous Schwann cells, there was no difference between the curves derived from endogenous SC's cultured alongside BFP-SKP-SC's (endoSKP-SCs), or endogenous Schwann cells cultured alongside BFP-Schwann cells (endoSchwanns) (2-way ANOVA, $\alpha 0.05$). Within an individual cell type, differences amongst PIs for days post myelin induction were often significant, again demonstrating a real effect of spectral change with time (2-way ANOVA, Tukey's post-test, Appendix Figure 2b). In general, the non-polar timepoints (Day 13, and day 22 for the endoSKP-SC myelin) were statistically different from the more polar timepoints. For endoSKP-SCs: Day 13, $n=19$; Day 16, $n=17$, Day 19, $n=31$; Day 22, $n=20$. For endoSCs: Day 13, $n=11$; Day 16, $n=12$; Day 19, $n=30$; Day 22, $n=16$.

Interestingly, there was a clear and significant difference between the total curves for BFP (exogenous) vs. GFP (endogenous) cells (SKP-SC > endoSKP-SC, Schwann > endoSchwann; 2-way ANOVA, $\alpha 0.05$).

6.4.2.3 Intravital analysis

Using our previously described tibial nerve window imaging technique, we were able to determine the myelin specific polarity index of cell grafts over sequential days in our doxorubicin injury model, again beginning at 21 days post injury (15 days post cell injection).

Aside from the quantification of these data, our nerve window technique gave beautiful visualization of the regenerating nerve environment (Figure 5-6).

Clearly demonstrated are GFP+ve axons with regenerating myelin, often as conferred by the BFP+ve cell grafts (Figure 5-6c, 5-7). Also present were varying degree of myelin debris, likely located within extratubal macrophages in the regenerating environment. On occasion these debris also contain BFP positivity, suggesting that some of the injected cells died and were phagocytosed.

The overall trend of biphasic polarity index progression was maintained for our intravital analysis of cell transplant derived myelin. Myelin selection regions for both SKP-SCs and Schwann cells had a relatively low PI, progressed through a period of higher PI, and returned to a state of lower PI with increasing maturity (Figure 6-8 A-D). In general, there was no significant difference between the SKP-SC and SC curves (2-way ANOVA, $\alpha 0.05$).

For a particular cell therapy, there were many statistical differences amongst myelin PI's of the timepoints measured, primarily between the least and most polar signatures observed, demonstrating a significant spectral change in myelin PI signature over time (summarized in Appendix). Finally, there was a significant difference between SKP-SC myelin PI and Schwann myelin PI at Day 27 (2-way ANOVA, $p < 0.001$).

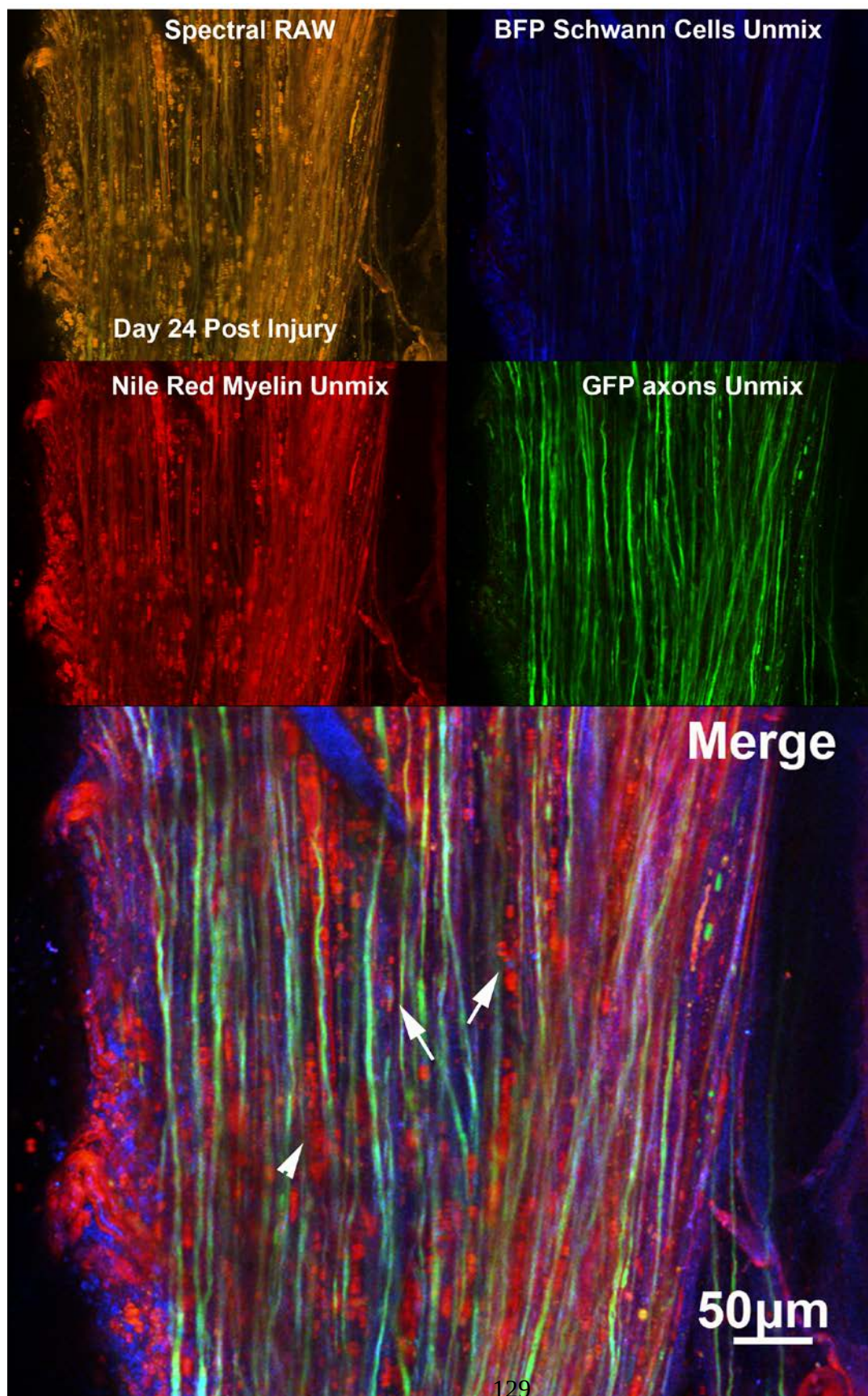


Figure 6-6 Intravital spectral imaging of a transplanted Schwann cell graft. Intravital image, widefield zoom of regenerating tibial nerve 24 days post doxorubicin injury, 15 days after injection of BFP+ve Schwann cell graft. A) Spectral A1R Screenshot B) BFP unmix (Schwann cells) C) Nile Red unmix (myelin) D) GFP unmix (axons) E) 3-channel spectral unmix, merge. 7-0 prolene stitch is visible in the top mid-left of the image (blue auto-fluorescence). Many Nile Red positive debris laden cells are present in the extratubular space, likely in macrophages (Arrowhead). Some contain BFP positive vacuoles surrounded by a Nile Red positive circumference, likely representative of dead BFP-SCs that have been phagocytosed (Arrows).

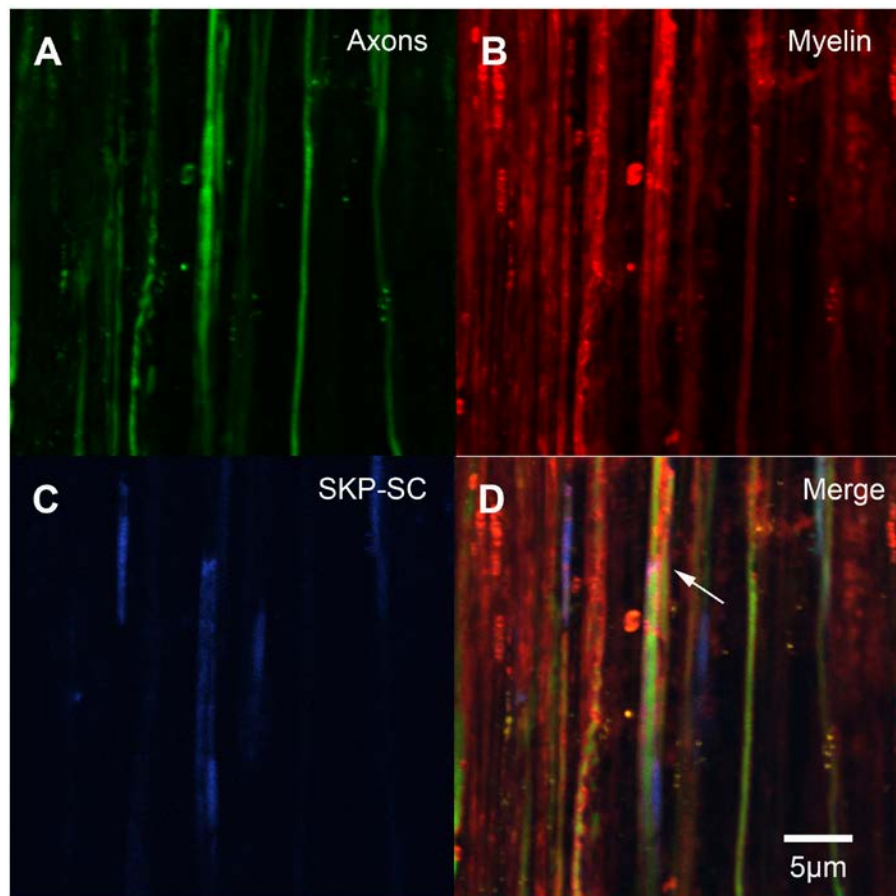


Figure 6-7 High magnification intravital image of BFP+ve SKP-SC myelination. Intravital tibial nerve image, high magnification 3-channel, demonstrating live myelination by SKP cells 27 days post doxorubicin injury, 19 days post cell injection. A) Green Channel = GFP axons B) Red Channel = Nile Red Myelin C) Blue Channel = BFP Schwann cells. Arrow = Node of Ranvier.

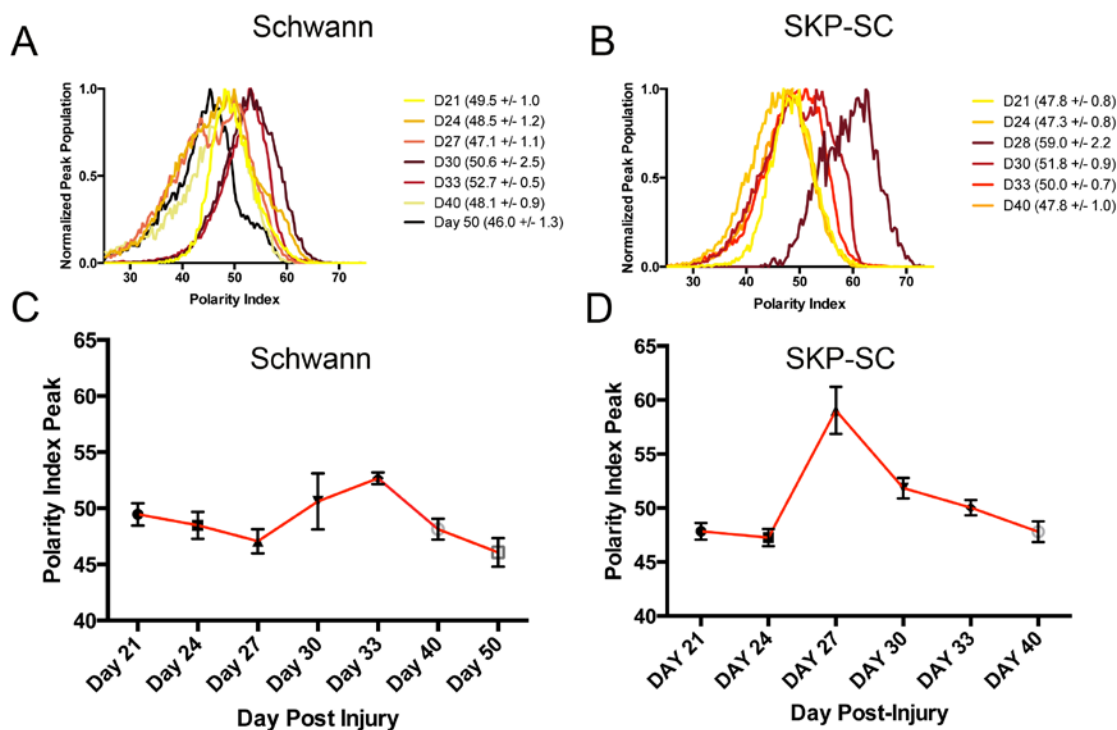


Figure 6-8 Intravital polarity index analysis of SKP-SCs and Schwann cell transplants over sequential days of myelination. A) Average Polarity Index curves for Schwann cell transplant myelin (Day 21, n=8; Day 24 n=28; Day 27 n=27, Day 30 n=20; day 40, n=15; day 50, n=17). B) SKP-SC PI average curves (Day 21, n=7; Day 24, n=28, Day 27, n=5, Day 30, n=31, Day 33, n=25, Day 40, n=15). Both data demonstrate a biphasic polarity index with time, moving from

an early blue shifted PI, through a red shifted PI, and back to a blue shifted PI at the most mature time points. C) Peak PI values vs. time, Schwann cells D) Peak PI values vs. time, SKP-SC cells.

6.5 Discussion

6.5.1 Schwann cells and SKP-SCs myelinate axons in-vivo and in-vitro

Our results demonstrate a strong myelinating capacity of for Schwann cells and SKP-SCs, both in-vitro and in-vivo. This is congruent with extensive literature detailing the known experimental behaviour of both of these cell types^{60,62,104,105,121,133,134,149}. Of note, we were able to use live imaging to capture peripheral nerve myelination by stem cell-derived Schwann cells, which is has not been previously published to our knowledge.

6.5.2 Regenerating myelin displays a biphasic progression in polarity index with increasing maturity.

We demonstrate using both in-vitro and in-vivo models that the myelination conferred by SKP-SC and Schwann cells follows a biphasic evolution of its chemical polarity index over time. As mentioned in Aim 1, we feel it is likely that blue shifts in polarity index (as measured by Nile Red) are due to increases in lipid order and lateral lipid packing, thereby representing an increased fraction of myelin membrane cholesterol. In this manner, our data suggest that the incorporation of cholesterol into regenerating myelin follows a different time course than that present during developmental sciatic myelination, which is known to accumulate slowly over the course of approximately 8 weeks¹⁷⁸.

The biochemistry of remyelination has been well studied in a group of seminal papers that used radiolabelling techniques to determine the proportional contributions of lipid de-novo synthesis, as compared to lipid recycling during regeneration of the injured PNS^{54-57,68,125,126}. Although these studies were initially performed after axonotmetic injury, the findings were later validated in a model of primary demyelination (tellurium)⁶⁸.

Fascinatingly, cholesterol of degenerated myelin is *almost entirely conserved*, and incorporated into the new myelin membrane from the outset of remyelination¹⁶⁹. Consistent with this, actual de-novo cholesterol synthesis is down-regulated⁵⁵ in Schwann cells in early Wallerian degeneration. The recycled cholesterol is formed into cholesterol esters by macrophages, whereby it associates with Apolipoprotein E⁵⁴ to become available for transport, and thereby interaction with LDL receptors inherent on Schwann cells during PNS regeneration⁹⁸. Myelin phospholipids are entirely degraded, with no recycling of the glycerol backbone⁵⁷. However, the fatty acid component of these phospholipids is recycled, with approximately 50% of degenerated fatty acids becoming incorporated into newly formed myelin membrane⁵⁷. Immediate incorporation of cholesterol into regenerating myelin is consistent with our data, which suggests that that early myelination likely displays lateral membrane density close to that of uninjured myelin.

Our data also consistently demonstrate a distinctive rise in polarity index after this point in time, always present to a greater or lesser degree. From a membrane packing standpoint, this would represent the incorporation of large amounts of phospholipid into the myelin. Interestingly, the study of alterations in P0 and MBP expression after crush injury in the rat suggest that these

genes are markedly down-regulated for the first 7 days following PNS injury⁶⁶. Following this, there is a period of up-regulation, with the sharpest increase of coding transcripts occurring 10-14 days after injury, and reaching a peak at 21 days after crush. Congruently, synthesis of new myelin phospholipid begin 5 days after Tellurium injury, and reaches a peak level plateau at 9 days (as evidenced by measured incorporation of radiolabeled [³H]glycerol into new sciatic nerve lipids)⁶⁸.

Finally, endogenous cholesterol production after Tellurium injury remains markedly low for the first nine days after injury, and then rises to control levels by approximately 15 days⁶⁸. This means that after a Tellurium demyelination, and perhaps after a demyelination injury in general, there is a period of time when a Schwann cell uses primarily recycled cholesterol (approx. 9 days) before switching metabolism to cholesterol production. Very likely this is due to a decrease in available imported/lipoprotein associated cholesterol levels up-regulating the function of HMG-CoA reductase⁸¹. As low levels of available cholesterol are required to stimulate cholesterol production by the Schwann cell, there is likely a transient lag in intracellular cholesterol levels. This lag takes place at very much the same that new phospholipid production is reaching an apex, judging by the published dynamics of these phenomena in remyelination from tellurium induced injury⁶⁸. We propose that the interplay between cholesterol reuse, phospholipid production, and endogenous cholesterol production forms basis of the spectral changes that we see with progressive myelin maturity. The idea is presented visually in Figure 6-9. Although we do see instances of both cell types comparatively moving through this progression at an expedited rate, it may be incorrect to assume that these dynamics represent a faster myelination “speed” by either SKP-SCs or SCs.

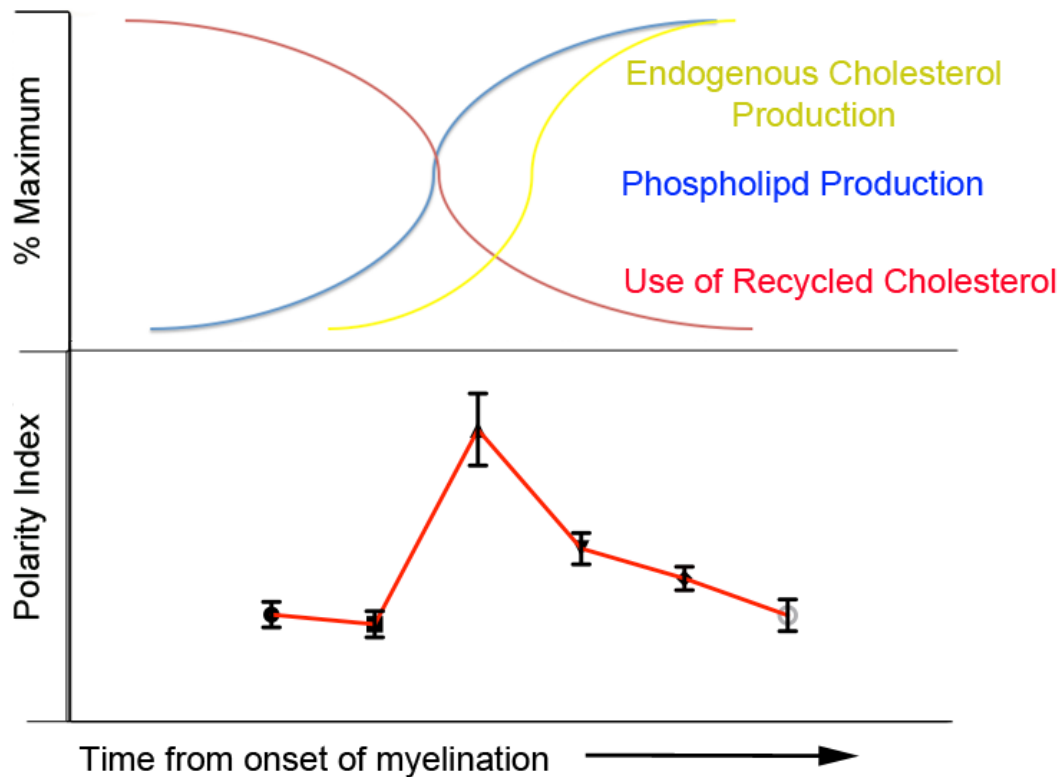


Figure 6-9 A theory regarding the biphasic evolution of myelin polarity index over early time-points of remyelination. Schwann cells initially and extensively incorporate salvaged cholesterol, presenting new myelin with a low polarity index. Upregulation of phospholipid production occurs during a nadir of cholesterol levels, while the cell is switching from exogenous cholesterol use to endogenous cholesterol production. The dominant addition of phospholipid to the myelin during this time results in a transient increase in the polarity index of myelin signatures. Endogenous cholesterol production then reaches full capacity and is gradually incorporated into the phospholipid rich myelin, resulting in a gradual return of myelin polarity index to lower levels. These processes occur over approximately two weeks.

What these curves likely represent is a variable degree of coordination of the aforementioned cellular processes, such that PI shifts appear to be earlier, more synchronous, or more stretched out depending on other yet unknown biologic conditions.

6.5.3 Different myelin conditions demonstrate vertical shifting of the biphasic curve.

Although each condition we tested demonstrated this biphasic polarity response to a greater or lesser degree, there are marked variations amongst the range of PI measurements over which these fluctuations occur.

Our data show that fixed tissue analysis yields a blue shifted PI curve, as compared to any of the live myelin analysis scenarios. An obvious explanation for this is that immobile molecules have greater opportunity to contribute stability to the excited state of a solvatochromic fluorophore⁹¹. However, it is also apparent that the in-vitro myelin of endogenous GFP Schwann cells is on average less polar than that of BFP positive cells introduced into the co-culture. It would appear that the effect is not secondary to the presence of the BFP fluorophore, as BFP myelin measured by intravital imaging has a similar range of PI when compared with the endogenous in-vitro GFP Schwann cell population. It may be that the close association of lipid and cholesterol rich cells (macrophages/fibroblasts) present within the DRG are able to confer some of this metabolic food to endogenous Schwann cells before they begin to myelinate, such that the difference in molecular starting blocks skew the curve of the endogenous cells. Schwann cells are well known to express LDL receptors, and receive lipid raw material from adjacent cells via an ApoE

mediated mechanism^{51,52,54}. Our in-vivo images often demonstrate extensive lipid rich deposits in Schwann cells, particularly in GFP positive endogenous cells, and particularly during early myelination time-points (data not shown).

A final observation about the in-vitro data is that the PI of both the SKP-SCs/SCs follows the dynamics of their respective endogenous co-culture condition, suggesting that a potentially diffusible factor may regulate or synchronize the metabolic processes suggested in Figure 9.

6.6 Conclusions

We have demonstrated that Nile Red emission spectra changes in a biphasic pattern when used to probe remyelination chemistry during the first two weeks of myelination, a finding that was consistent in each experimental paradigm we tested. The changes in polarity index demonstrated are plausible, in the context of what is known about the chemistry of early remyelination in the PNS following injury.

The potential ability of Nile Red to indicate (within a 48hr window) the “phase” of myelin regeneration taking place is a unique and powerful tool for the analysis of regenerative phenomena, in vitro as well as in-vivo. Our observations suggested that a potential diffusible factor(s) may be responsible for coordinating biochemical pathways of regenerating myelin in-vitro; myelin spectroscopy by Nile Red PI analysis provides an experimental tool to test that hypothesis with. Furthermore, questions about the general regenerative progression of in-vivo remyelination may now be amenable to intravital analysis, with wide applicability to study of PNS, and very likely CNS injury phenomena.

6.7 Appendix: Chapter 6 Statistical Analysis

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Day 21:Schwann vs. Day 21:SKP-SC	3.38	-0.4158 to 7.177	No	ns
Day 21:Schwann vs. Day 24:Schwann	-2.302	-5.919 to 1.315	No	ns
Day 21:Schwann vs. Day 24:SKP-SC	-0.7038	-4.500 to 3.092	No	ns
Day 21:Schwann vs. Day 27:Schwann	-3.646	-7.777 to 0.4856	No	ns
Day 21:Schwann vs. Day 27:SKP-SC	-4.596	-8.392 to -0.7999	Yes	**
Day 21:Schwann vs. Day 30:Schwann	-4.009	-7.985 to -0.03305	Yes	*
Day 21:Schwann vs. Day 30:SKP-SC	-3.745	-7.346 to -0.1449	Yes	*
Day 21:Schwann vs. Day 33:Schwann	1.386	-2.688 to 5.460	No	ns
Day 21:Schwann vs. Day 33:SKP-SC	-1.914	-5.456 to 1.628	No	ns
Day 21:Schwann vs. Adult:Schwann	0.7312	-3.943 to 5.405	No	ns
Day 21:Schwann vs. Adult:SKP-SC	0.7312	-3.943 to 5.405	No	ns
Day 21:SKP-SC vs. Day 24:Schwann	-5.682	-8.730 to -2.634	Yes	****
Day 21:SKP-SC vs. Day 24:SKP-SC	-4.084	-7.343 to -0.8256	Yes	**
Day 21:SKP-SC vs. Day 27:Schwann	-7.026	-10.67 to -3.383	Yes	****
Day 21:SKP-SC vs. Day 27:SKP-SC	-7.977	-11.24 to -4.718	Yes	****
Day 21:SKP-SC vs. Day 30:Schwann	-7.389	-10.86 to -3.923	Yes	****
Day 21:SKP-SC vs. Day 30:SKP-SC	-7.126	-10.15 to -4.097	Yes	****
Day 21:SKP-SC vs. Day 33:Schwann	-1.995	-5.573 to 1.584	No	ns
Day 21:SKP-SC vs. Day 33:SKP-SC	-5.295	-8.253 to -2.336	Yes	****
Day 21:SKP-SC vs. Adult:Schwann	-2.649	-6.898 to 1.599	No	ns
Day 21:SKP-SC vs. Adult:SKP-SC	-2.649	-6.898 to 1.599	No	ns
Day 24:Schwann vs. Day 24:SKP-SC	1.598	-1.450 to 4.646	No	ns
Day 24:Schwann vs. Day 27:Schwann	-1.344	-4.800 to 2.113	No	ns
Day 24:Schwann vs. Day 27:SKP-SC	-2.294	-5.343 to 0.7539	No	ns
Day 24:Schwann vs. Day 30:Schwann	-1.707	-4.977 to 1.562	No	ns
Day 24:Schwann vs. Day 30:SKP-SC	-1.444	-4.244 to 1.357	No	ns
Day 24:Schwann vs. Day 33:Schwann	3.688	0.2996 to 7.075	Yes	*
Day 24:Schwann vs. Day 33:SKP-SC	0.3875	-2.338 to 3.113	No	ns
Day 24:Schwann vs. Adult:Schwann	3.033	-1.057 to 7.123	No	ns

Day 24:Schwann vs. Adult:SKP-SC	3.033	-1.057 to 7.123	No	ns
Day 24:SKP-SC vs. Day 27:Schwann	-2.942	-6.585 to 0.7015	No	ns
Day 24:SKP-SC vs. Day 27:SKP-SC	-3.892	-7.151 to -0.6337	Yes	**
Day 24:SKP-SC vs. Day 30:Schwann	-3.305	-6.772 to 0.1611	No	ns
Day 24:SKP-SC vs. Day 30:SKP-SC	-3.042	-6.070 to -0.01323	Yes	*
Day 24:SKP-SC vs. Day 33:Schwann	2.089	-1.489 to 5.668	No	ns
Day 24:SKP-SC vs. Day 33:SKP-SC	-1.211	-4.169 to 1.748	No	ns
Day 24:SKP-SC vs. Adult:Schwann	1.435	-2.814 to 5.684	No	ns
Day 24:SKP-SC vs. Adult:SKP-SC	1.435	-2.814 to 5.684	No	ns
Day 27:Schwann vs. Day 27:SKP-SC	-0.9506	-4.594 to 2.693	No	ns
Day 27:Schwann vs. Day 30:Schwann	-0.3635	-4.194 to 3.467	No	ns
Day 27:Schwann vs. Day 30:SKP-SC	-0.09978	-3.539 to 3.339	No	ns
Day 27:Schwann vs. Day 33:Schwann	5.031	1.099 to 8.963	Yes	**
Day 27:Schwann vs. Day 33:SKP-SC	1.731	-1.646 to 5.109	No	ns
Day 27:Schwann vs. Adult:Schwann	4.377	-0.1738 to 8.927	No	ns
Day 27:Schwann vs. Adult:SKP-SC	4.377	-0.1738 to 8.927	No	ns
Day 27:SKP-SC vs. Day 30:Schwann	0.5871	-2.879 to 4.053	No	ns
Day 27:SKP-SC vs. Day 30:SKP-SC	0.8508	-2.178 to 3.879	No	ns
Day 27:SKP-SC vs. Day 33:Schwann	5.982	2.403 to 9.560	Yes	****
Day 27:SKP-SC vs. Day 33:SKP-SC	2.682	-0.2768 to 5.640	No	ns
Day 27:SKP-SC vs. Adult:Schwann	5.327	1.079 to 9.576	Yes	**
Day 27:SKP-SC vs. Adult:SKP-SC	5.327	1.079 to 9.576	Yes	**
Day 30:Schwann vs. Day 30:SKP-SC	0.2637	-2.987 to 3.515	No	ns
Day 30:Schwann vs. Day 33:Schwann	5.395	1.626 to 9.163	Yes	***
Day 30:Schwann vs. Day 33:SKP-SC	2.095	-1.091 to 5.281	No	ns
Day 30:Schwann vs. Adult:Schwann	4.74	0.3301 to 9.150	Yes	*
Day 30:Schwann vs. Adult:SKP-SC	4.74	0.3301 to 9.150	Yes	*
Day 30:SKP-SC vs. Day 33:Schwann	5.131	1.761 to 8.501	Yes	****
Day 30:SKP-SC vs. Day 33:SKP-SC	1.831	-0.8718 to 4.534	No	ns
Day 30:SKP-SC vs. Adult:Schwann	4.476	0.4017 to 8.551	Yes	*
Day 30:SKP-SC vs. Adult:SKP-SC	4.476	0.4017 to 8.551	Yes	*
Day 33:Schwann vs. Day 33:SKP-SC	-3.3	-6.608 to 0.007495	No	ns
Day 33:Schwann vs. Adult:Schwann	-0.6546	-5.153 to 3.844	No	ns
Day 33:Schwann vs. Adult:SKP-SC	-0.6546	-5.153 to 3.844	No	ns

Day 33:SKP-SC vs. Adult:Schwann	2.645	-1.378 to 6.669	No	ns
Day 33:SKP-SC vs. Adult:SKP-SC	2.645	-1.378 to 6.669	No	ns
Adult:Schwann vs. Adult:SKP-SC	0	-5.048 to 5.048	No	ns

Table 1 Two way-ANOVA data ($\alpha=0.05$) comparing the myelin PI from Figure 1C, by cell type and post-injury day (Fixed tissue analysis).

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
		0.3461 to		
Day 13:SKP-SC vs. Day 13:Schwann	5.4	10.45	Yes	*
		-13.08 to -		
Day 13:SKP-SC vs. Day 16:SKP-SC	-8.499	3.918	Yes	****
		-14.64 to -		
Day 13:SKP-SC vs. Day 16:Schwann	-9.268	3.896	Yes	****
		-11.92 to -		
Day 13:SKP-SC vs. Day 19:SKP-SC	-6.545	1.173	Yes	**
		-11.76 to -		
Day 13:SKP-SC vs. Day 19:Schwann	-7.151	2.540	Yes	***
Day 13:SKP-SC vs. Day 22:SKP-SC	-2.728	-7.721 to 2.264	No	ns
		-13.55 to -		
Day 13:SKP-SC vs. Day 22:Schwann	-8.91	4.268	Yes	****
		-18.40 to -		
Day 13:Schwann vs. Day 16:SKP-SC	-13.9	9.394	Yes	****
		-19.97 to -		
Day 13:Schwann vs. Day 16:Schwann	-14.67	9.361	Yes	****
		-17.25 to -		
Day 13:Schwann vs. Day 19:SKP-SC	-11.94	6.638	Yes	****
		-17.09 to -		
Day 13:Schwann vs. Day 19:Schwann	-12.55	8.016	Yes	****
Day 13:Schwann vs. Day 22:SKP-SC	-8.128	-13.05 to -	Yes	****

		3.206			
		-18.88 to -			
Day 13:Schwann vs. Day 22:Schwann	-14.31	9.743	Yes	****	
Day 16:SKP-SC vs. Day 16:Schwann	-0.7687	-5.628 to 4.090	No	ns	
Day 16:SKP-SC vs. Day 19:SKP-SC	1.954	-2.904 to 6.813	No	ns	
Day 16:SKP-SC vs. Day 19:Schwann	1.348	-2.653 to 5.350	No	ns	
Day 16:SKP-SC vs. Day 22:SKP-SC	5.771	1.335 to 10.21	Yes	**	
Day 16:SKP-SC vs. Day 22:Schwann	-0.411	-4.448 to 3.627	No	ns	
Day 16:Schwann vs. Day 19:SKP-SC	2.723	-2.887 to 8.334	No	ns	
Day 16:Schwann vs. Day 19:Schwann	2.117	-2.770 to 7.004	No	ns	
Day 16:Schwann vs. Day 22:SKP-SC	6.539	1.291 to 11.79	Yes	**	
Day 16:Schwann vs. Day 22:Schwann	0.3577	-4.559 to 5.274	No	ns	
Day 19:SKP-SC vs. Day 19:Schwann	-0.606	-5.493 to 4.281	No	ns	
Day 19:SKP-SC vs. Day 22:SKP-SC	3.816	-1.432 to 9.064	No	ns	
Day 19:SKP-SC vs. Day 22:Schwann	-2.365	-7.282 to 2.551	No	ns	
		-0.04383 to			
Day 19:Schwann vs. Day 22:SKP-SC	4.422	8.888	No	ns	
Day 19:Schwann vs. Day 22:Schwann	-1.759	-5.830 to 2.312	No	ns	
		-10.68 to -			
Day 22:SKP-SC vs. Day 22:Schwann	-6.182	1.683	Yes	**	

Table 2 2-way ANOVA ($\alpha=0.05$) comparing the PIs of in-vitro BFP-SKP-SC and BFP-SCs as a function of cell type and post-myelin induction day.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Day 13:EndoSKP-SC vs. Day 13:EndoSCs	-0.4765	-7.465 to 6.512	No	ns
		-20.25 to -		
Day 13:EndoSKP-SC vs. Day 16:EndoSKP-SC	-14.1	7.938	Yes	****
Day 13:EndoSKP-SC vs. Day 16:EndoSCs	-14.35	-21.15 to -	Yes	****

		7.548			
		-14.19 to -			
Day 13:EndoSKP-SC vs. Day 19:EndoSKP-SC	-8.814	3.440	Yes	****	
		-21.67 to -			
Day 13:EndoSKP-SC vs. Day 19:EndoSCs	-16.27	10.86	Yes	****	
		-12.90 to -			
Day 13:EndoSKP-SC vs. Day 22:EndoSKP-SC	-6.993	1.084	Yes	**	
		-20.80 to -			
Day 13:EndoSKP-SC vs. Day 22:EndoSCs	-14.54	8.282	Yes	****	
		-20.76 to -			
Day 13:EndoSCs vs. Day 16:EndoSKP-SC	-13.62	6.482	Yes	****	
		-21.57 to -			
Day 13:EndoSCs vs. Day 16:EndoSCs	-13.87	6.173	Yes	****	
		-14.81 to -			
Day 13:EndoSCs vs. Day 19:EndoSKP-SC	-8.338	1.864	Yes	**	
		-22.29 to -			
Day 13:EndoSCs vs. Day 19:EndoSCs	-15.79	9.288	Yes	****	
		-13.44 to			
Day 13:EndoSCs vs. Day 22:EndoSKP-SC	-6.517	0.4075	No	ns	
		-21.29 to -			
Day 13:EndoSCs vs. Day 22:EndoSCs	-14.06	6.840	Yes	****	
Day 16:EndoSKP-SC vs. Day 16:EndoSCs	-0.2527	-7.207 to 6.702	No	ns	
		-0.2848 to			
Day 16:EndoSKP-SC vs. Day 19:EndoSKP-SC	5.282	10.85	No	ns	
Day 16:EndoSKP-SC vs. Day 19:EndoSCs	-2.169	-7.769 to 3.430	No	ns	
Day 16:EndoSKP-SC vs. Day 22:EndoSKP-SC	7.103	1.018 to 13.19	Yes	*	
Day 16:EndoSKP-SC vs. Day 22:EndoSCs	-0.4443	-6.869 to 5.981	No	ns	
		-0.7365 to			
Day 16:EndoSCs vs. Day 19:EndoSKP-SC	5.535	11.81	No	ns	
Day 16:EndoSCs vs. Day 19:EndoSCs	-1.917	-8.217 to 4.384	No	ns	
Day 16:EndoSCs vs. Day 22:EndoSKP-SC	7.356	0.6206 to 14.09	Yes	*	
Day 16:EndoSCs vs. Day 22:EndoSCs	-0.1916	-7.236 to 6.853	No	ns	
		-12.18 to -			
Day 19:EndoSKP-SC vs. Day 19:EndoSCs	-7.451	2.727	Yes	****	

Day 19:EndoSKP-SC vs. Day 22:EndoSKP-SC	1.821	-3.469 to 7.112	No	ns
		-11.40 to -		
Day 19:EndoSKP-SC vs. Day 22:EndoSCs	-5.726	0.04826	Yes	*
Day 19:EndoSCs vs. Day 22:EndoSKP-SC	9.273	3.948 to 14.60	Yes	****
Day 19:EndoSCs vs. Day 22:EndoSCs	1.725	-3.985 to 7.435	No	ns
		-13.73 to -		
Day 22:EndoSKP-SC vs. Day 22:EndoSCs	-7.548	1.361	Yes	**

Table 3: 2-way ANOVA ($\alpha=0.05$) comparing the PIs of in-vitro endoSKP-SC and endoBFP-SCs as a function of cell type and post-myelin induction day.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Day 21:SKP-SC vs. Day 21:Schwann cells	-1.62	-10.86 to 7.624	No	ns
Day 21:SKP-SC vs. Day 24:SKP-SC	0.5786	-6.969 to 8.126	No	ns
Day 21:SKP-SC vs. Day 24:Schwann cells	-0.6393	-8.187 to 6.908	No	ns
		-21.66 to -		
Day 21:SKP-SC vs. Day 27:SKP-SC	-11.2	0.7391	Yes	*
Day 21:SKP-SC vs. Day 27:Schwann cells	0.7762	-6.799 to 8.352	No	ns
Day 21:SKP-SC vs. Day 30:SKP-SC	-3.993	-11.47 to 3.481	No	ns
Day 21:SKP-SC vs. Day 30:Schwann cells	-2.767	-10.61 to 5.076	No	ns
Day 21:SKP-SC vs. Day 33:SKP-SC	-2.192	-9.829 to 5.446	No	ns
Day 21:SKP-SC vs. Day 33:Schwann cells	-4.831	-12.31 to 2.643	No	ns
Day 21:SKP-SC vs. Day 40:SKP-SC	0.04952	-8.126 to 8.225	No	ns
Day 21:SKP-SC vs. Day 40:Schwann cells	-0.3009	-8.476 to 7.875	No	ns
Day 21:Schwann cells vs. Day 24:SKP-SC	2.198	-4.962 to 9.358	No	ns
Day 21:Schwann cells vs. Day 24:Schwann cells	0.9804	-6.180 to 8.141	No	ns
		-19.76 to		
Day 21:Schwann cells vs. Day 27:SKP-SC	-9.577	0.6046	No	ns
Day 21:Schwann cells vs. Day 27:Schwann cells	2.396	-4.794 to 9.585	No	ns
Day 21:Schwann cells vs. Day 30:SKP-SC	-2.373	-9.456 to 4.710	No	ns

Day 21:Schwann cells vs. Day 30:Schwann cells	-1.147	-8.619 to 6.324	No	ns
Day 21:Schwann cells vs. Day 33:SKP-SC	-0.5721	-7.827 to 6.683	No	ns
Day 21:Schwann cells vs. Day 33:Schwann cells	-3.212	-10.29 to 3.871	No	ns
Day 21:Schwann cells vs. Day 40:SKP-SC	1.669	-6.150 to 9.488	No	ns
Day 21:Schwann cells vs. Day 40:Schwann cells	1.319	-6.501 to 9.138	No	ns
Day 24:SKP-SC vs. Day 24:Schwann cells	-1.218	-5.991 to 3.556	No	ns
		-20.45 to -		
Day 24:SKP-SC vs. Day 27:SKP-SC	-11.78	3.104	Yes	***
Day 24:SKP-SC vs. Day 27:Schwann cells	0.1976	-4.620 to 5.015	No	ns
		-9.228 to		
Day 24:SKP-SC vs. Day 30:SKP-SC	-4.571	0.08532	No	ns
Day 24:SKP-SC vs. Day 30:Schwann cells	-3.346	-8.575 to 1.883	No	ns
Day 24:SKP-SC vs. Day 33:SKP-SC	-2.77	-7.685 to 2.144	No	ns
		-10.07 to -		
Day 24:SKP-SC vs. Day 33:Schwann cells	-5.41	0.7534	Yes	**
Day 24:SKP-SC vs. Day 40:SKP-SC	-0.529	-6.244 to 5.186	No	ns
Day 24:SKP-SC vs. Day 40:Schwann cells	-0.8795	-6.594 to 4.835	No	ns
		-19.23 to -		
Day 24:Schwann cells vs. Day 27:SKP-SC	-10.56	1.887	Yes	**
Day 24:Schwann cells vs. Day 27:Schwann cells	1.415	-3.402 to 6.233	No	ns
Day 24:Schwann cells vs. Day 30:SKP-SC	-3.353	-8.010 to 1.303	No	ns
Day 24:Schwann cells vs. Day 30:Schwann cells	-2.128	-7.357 to 3.101	No	ns
Day 24:Schwann cells vs. Day 33:SKP-SC	-1.552	-6.467 to 3.362	No	ns
		-8.849 to		
Day 24:Schwann cells vs. Day 33:Schwann cells	-4.192	0.4645	No	ns
Day 24:Schwann cells vs. Day 40:SKP-SC	0.6888	-5.026 to 6.404	No	ns
Day 24:Schwann cells vs. Day 40:Schwann cells	0.3384	-5.376 to 6.053	No	ns
Day 27:SKP-SC vs. Day 27:Schwann cells	11.97	3.278 to 20.67	Yes	***
Day 27:SKP-SC vs. Day 30:SKP-SC	7.205	-1.403 to 15.81	No	ns
		-0.5003 to		
Day 27:SKP-SC vs. Day 30:Schwann cells	8.43	17.36	No	ns
Day 27:SKP-SC vs. Day 33:SKP-SC	9.005	0.2555 to 17.76	Yes	*
Day 27:SKP-SC vs. Day 33:Schwann cells	6.366	-2.242 to 14.97	No	ns
Day 27:SKP-SC vs. Day 40:SKP-SC	11.25	2.024 to 20.47	Yes	**

Day 27:SKP-SC vs. Day 40:Schwann cells	10.9	1.673 to 20.12	Yes	**
		-9.470 to -		
Day 27:Schwann cells vs. Day 30:SKP-SC	-4.769	0.06721	Yes	*
Day 27:Schwann cells vs. Day 30:Schwann cells	-3.543	-8.813 to 1.726	No	ns
Day 27:Schwann cells vs. Day 33:SKP-SC	-2.968	-7.925 to 1.989	No	ns
		-10.31 to -		
Day 27:Schwann cells vs. Day 33:Schwann cells	-5.608	0.9059	Yes	**
Day 27:Schwann cells vs. Day 40:SKP-SC	-0.7267	-6.478 to 5.025	No	ns
Day 27:Schwann cells vs. Day 40:Schwann cells	-1.077	-6.829 to 4.675	No	ns
Day 30:SKP-SC vs. Day 30:Schwann cells	1.225	-3.897 to 6.348	No	ns
Day 30:SKP-SC vs. Day 33:SKP-SC	1.801	-3.000 to 6.602	No	ns
Day 30:SKP-SC vs. Day 33:Schwann cells	-0.8387	-5.375 to 3.698	No	ns
Day 30:SKP-SC vs. Day 40:SKP-SC	4.042	-1.575 to 9.660	No	ns
Day 30:SKP-SC vs. Day 40:Schwann cells	3.692	-1.926 to 9.309	No	ns
Day 30:Schwann cells vs. Day 33:SKP-SC	0.5754	-4.783 to 5.934	No	ns
Day 30:Schwann cells vs. Day 33:Schwann cells	-2.064	-7.187 to 3.058	No	ns
Day 30:Schwann cells vs. Day 40:SKP-SC	2.817	-3.284 to 8.917	No	ns
Day 30:Schwann cells vs. Day 40:Schwann cells	2.466	-3.634 to 8.567	No	ns
Day 33:SKP-SC vs. Day 33:Schwann cells	-2.64	-7.441 to 2.161	No	ns
Day 33:SKP-SC vs. Day 40:SKP-SC	2.241	-3.592 to 8.075	No	ns
Day 33:SKP-SC vs. Day 40:Schwann cells	1.891	-3.942 to 7.724	No	ns
		-0.7367 to		
Day 33:Schwann cells vs. Day 40:SKP-SC	4.881	10.50	No	ns
Day 33:Schwann cells vs. Day 40:Schwann cells	4.53	-1.087 to 10.15	No	ns
Day 40:SKP-SC vs. Day 40:Schwann cells	-0.3504	-6.872 to 6.171	No	ns

Table 4 Two way-ANOVA data ($\alpha=0.05$) comparing the myelin PI from Figure 5 (Intravital analysis), by cell type and post-injury day.

Chapter Seven: **Discussion**

7.1 General Discussion

Although this dissertation consists of several overarching and interconnected goals, it began with some key questions. Are solvatochromic dyes effective tools to indicate known differences in PNS myelin? If so, which dye is best? And given this, if these dyes are intimately sensitive to membrane chemistry, can we use them to study living myelin? Can they be used to provide some clue as to the maturity of newly forming myelin, whereby such information would previously require animal sacrifice and tissue processing? These queries formed the basis of our investigation.

It is well known that solvatochromism is a property inherent in ICT class membrane dyes, which has been extensively exploited for experimental membrane study^{3,32,59,88,89,112}. We first aimed to demonstrate that solvatochromic effects were applicable and generalizable to the study of PNS myelin. Besides the parallel work being done to study the effects of solvatochromism in CNS tissue by Teo and Stys (unpublished), no previous work to date has detailed the use of these indicators in the context of myelin spectroscopy. Vital dyes (dyes that can be used to stain living tissue) such as fluoromyelin have indeed been used previously as stains for living myelin^{109,130}, but for anatomical contrast only.

In Chapter Three we show that not all dyes are equally effective for the purposes of PNS myelin analysis. We evaluated four separate dyes extensively for reliability, signal strength, stability, and overall ability to detect known differences in myelin composition across various developmental ages. We further tested several dyes for efficacy in differentiating mutant myelin

from wildtype myelin, using Shiverer and SCAP dysmyelination mutant strains as our “altered” myelin of choice. After this robust field-testing, we demonstrated that Nile Red is superior to other dyes for its consistency and sensitivity, making this dye the focal point of all of our further analysis.

Our desire to use this dye to probe the maturity of PNS remyelination also presented with a host of challenges. Studies of remyelination are difficult to perform for several reasons. On a basic level it can be difficult to distinguish a remyelinated axon from an uninjured axon. There are several hallmark features of a remyelinated axon; namely, the myelin thickness is too thin for the axon calibre, and the internodal length is shortened¹⁴. However, the parameter of altered myelin thickness is only applicable in the analysis of a subset of large diameter axons, while the measurement of internodal length requires longitudinal histologic analysis¹⁴. Furthermore, the more mature that a remyelinated axon becomes, the more it resembles an axon that has never been injured. Add these confounders to the fact that most demyelination type injuries are often variable from subject to subject, we can see how remyelination analysis may pose distinct methodological issues. Many investigators have turned to systemic toxin-induced demyelination models, thereby inducing complete and uniform demyelination injury^{68,101,140,155}. While convenient for experimental purposes, these injuries may fail to mirror the pathological mechanisms and conditions of diseases such as multiple sclerosis, where the location and severity of demyelinating plaques may change with time.

Considering the above, we decided to analyze remyelination spectra by focusing on myelin provided by fluorescently labeled cell grafts. In this manner, we could be assured that the myelin

we were quantifying was indeed regenerating. In Chapter Four we resurrect a model of focal demyelination using doxorubicin³⁷, an injury model with an extended 40-50 day time-course of demyelination followed by remyelination. Unlike models of demyelination where lesions are spatially disparate and variable, this model permits us to choose and serially evaluate a known area of demyelination. The kinetics are also such that a properly timed cell graft transplant actually has the opportunity to integrate with axons during the maxima of endogenous Schwann cell loss. Not only do we show that SKP-SCs can form mature myelin in this model, but through the use of labeled cell grafts and solvatochromic myelin dyes, we demonstrate a reliable method to identify myelin figures specifically derived from transplanted cells. This technology would form the basis upon which we could advance our investigation into remyelination spectral phenomena.

Living animal experiments are likely the realm in which the Nile Red solvatochromic assay has the greatest potential to provide unique scientific contributions over existing methods of myelin analysis. Another key challenge was therefore the development of a live imaging technique capable of analyzing the above injury model in a high-resolution spectral manner. As almost a project onto its own, Chapter 5 details our trials and efforts in creating this methodology. By the conclusion, we have surmounted many of the obstacles inherent in PNS intravital microscopy. We present a nerve window imaging technique that is stable enough to image the living PNS of the rat at micron resolution, and compatible with the long acquisition times necessary for high definition spectral imaging.

With all of these foundations in place, we became able to approach the investigation of regenerating myelin spectra. In Chapter six we examine regenerating myelin provided by SKP-SCs and Schwann cells, both in vitro, with DRG and Schwann/SKP-SC co-culture, and in vivo. Our in-vivo analysis included two separate reiterations of the doxorubicin injury/cell graft transplant model; one in which nerves were sacrificed at sequential time points and analyzed as fixed tissue specimens, and another in which nerves were analyzed using our live imaging nerve window technique. In a congruent fashion, all three paradigms of remyelination (and both cell types) demonstrated a consistent pattern of myelin specific polarity index changes, displayed over the first two weeks of new myelin formation. This biphasic pattern is likely representative of the progressive internal chemistry of the regenerating myelin sheath, as it fluctuates in cholesterol and phospholipid content, moving through lipid ordered and lipid disordered states.

7.2 Limitations

Our ability to comment on the chemistry of myelin is unfortunately limited to phenomenology when using this technique, at least for now. The actual linkage of myelin spectral data to the hard chemistry of imaged myelin is a future goal of this work. In essence we have performed the biological feasibility portion of this endeavour, demonstrating that solvatochromic analysis is indeed useful in the study of PNS myelin biology. Using our technique, we have also shown the unique ability to approach myelin analysis in novel situations, such as the study of living stem cell derived myelin. These works justify further study of the chemistry involved. Correlative studies using Nile Red spectroscopic analysis and high-resolution mass spectroscopic techniques such as MALDI¹⁶⁴ would be one possible means to approach this problem. Other experiments may include spectral observation during selective depletion and loading of key membrane

components (such as cholesterol) within our established in-vitro model system. Previous authors have used simple Nile Red component spectra to elucidate the proportional constituents of liposomes, using spectral deconvolution algorithms³⁴. Although of a far greater complexity, it may be possible to elucidate the component spectra of the common states of myelin, through injury and health, by correlating these spectra with hard chemical analysis. In this fashion, we may eventually be able to tell the exact composition of Nile Red stained myelin, simply by looking at the emission spectra.

As detailed as our study of spectral phenomena in the PNS has been, it has been limited to not only a single dye, but also to a single parameter of the emission characteristics of that dye (peak emission frequency). Other emission characteristics, such as the width of the emission spectra, may yield valuable information regarding the chemical environment of the dye. For example, one such technique termed “red edge excitation” describes the difference in Nile Red emission when stimulated by lasers of increasingly lower frequency¹¹⁹. This difference is directly proportional to the lipid order of the labeled membrane, and could be a useful correlate for our Polarity Index measurements. Furthermore, solvatochromic fluorophores can and do demonstrate alterations in absorption spectra⁹⁷; simply due to time constraints this entire avenue of inquiry has been bypassed in our current work.

Our work has also focused on the study of peripheral nervous system myelin. Although it is tempting to generalize our findings to the central nervous system, CNS myelin is different in a myriad of ways. It is produced by a different cell type, contains different membrane proteins,

and has a different periodicity than PNS myelin. It is very possible that the cellular machinery responsible for the spectral phenomena we have observed in the PNS work in a different manner for central myelin repair.

Despite our best effort, we were unable to image myelin at a depth of greater than 30-40 μ m in living tibial nerve. Our intravital results were therefore biased towards the analysis of cells with a preference for the periphery of a regenerative nerve environment. It is difficult to confirm whether this subpopulation is an accurate representation of cell graft behaviour in total; we have made the assumption that it is. The challenges of imaging at depth through thick myelin is confirmed in other reports^{35,41}.

Furthermore, we were unable to reliably demonstrate a method for chronic in-vivo PNS live imaging. We are confident that given enough time we could overcome this technical hurdle. At the extent of our trials, the current limitation to effective longitudinal study was an inflammatory reaction induced by the glass of the nerve window coverslip. By changing this interface to a biocompatible one, and perhaps by sequestering the nerve within a nerve regeneration tube¹⁶³, we could likely bypass this obstacle. A promising future direction would be to integrate our design with an external interface¹⁹, such that dissection would be unnecessary for repeated imaging sessions.

7.3 Conclusions and Future Directions

There are several major conclusions to be drawn from this thesis. One is that Nile Red is an effective fluorescent tool for probing known differences in PNS myelin composition. This

effectiveness is rooted in the solvatochromic properties of the dye. Another conclusion is that by using this new method of myelin analysis, we can comment on myelin chemistry in previously inaccessible scenarios, such as live imaging experiments or living in-vitro culture. Consistent with what is known regarding the biochemistry of early remyelination, the results of our experiments indicate that remyelination follows a biphasic chemical pattern that is reliably indicated by Nile Red polarity index analysis.

In developing this technology we have also made evident other important and noteworthy contributions. SKP-SCs myelinate axons in-vivo in a robust and physiologic manner, and do appear to exert positive effects on endogenous remyelination. SKP-SCs may also exert a paracrine effect in-vitro and in-vivo that coordinates the biochemical processes of neighbouring Schwann cells, though this evidence is quite preliminary. In general it would appear that the biochemical processes taken by SKP-SC to myelinate axons are very similar to those taken by Schwann cells, providing further evidence that these cells do in fact function as fully capable Schwann cell replacements. We have also adapted a reliable model of doxorubicin injury whereby many effects of cell transplantation can be studied in the presence of naked axons. Furthermore, by taking inspiration from the spinal cord window imaging literature⁴¹, we have invented and adapted a nerve imaging technique that allows for micron scale resolution and reliable spectral imaging.

Our hope would be for this work to translate into effective research tools for the investigation of myelin repair. With a firm grasp on the biphasic spectral response of myelin during regeneration, simple G-ratio calculations (a mainstay of regenerative study outcomes) could be replaced or

enhanced by automated spectral analysis. This analysis could quickly and accurately characterize the extent and biochemical state of regenerating myelin, without the aforementioned complications inherent when studying a demyelinated specimen. Furthermore, this analysis could also be performed in living animals, and with a little work, behavioural and solvatochromic readouts of nerve regeneration experiments could be run on the same rat simultaneously and over time.

Expanding the scope of applicability beyond PNS regeneration, this technique may fall into the limited category of experimental readouts that may be pertinent to the study of living humans. As a non-invasive spectroscopic tool, we may realize a utility for operating room scenarios as technology in general matures, microscopes miniaturize, motion-reduction software improves, and data-handling capacity expands. To be certain, the ability to comment of the chemical state of a single piece of myelin, in a non-invasive fashion, in any manner whatsoever, is a profound step forward from existing methods for the study of this fascinating tissue.

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