INVESTIGATION OF 1450 NM INFRARED LIGHT FOR CLINICAL NERVE

STIMULATION

By

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CHAPTER 1

INTRODUCTION

Overview of the Peripheral Nervous System

The peripheral nervous system (PNS), shown in Figure 1, is one of two parts of the nervous system in whole, the other being the central nervous system (CNS). The PNS transmits information via the transduction of numerous stimuli (tactile, proprioceptive, nociceptive, etc.) into electro-chemical signals. The PNS is composed of neurons, the functional unit of the nervous system, in addition to support cells and structures such as Schwann cells, which function as the primary glial cells of the PNS [1].
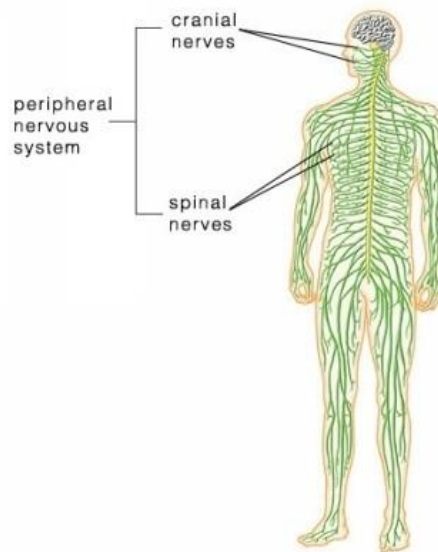

Figure 1: Illustration of the peripheral nervous system.

The PNS is capable of conducting both afferent and efferent information to and from the CNS. Afferent or sensory information is conducted through sensory neurons to the CNS, allowing for

the perception of inputs from outside stimuli. Efferent or motor information is conducted from the CNS through motor neurons, allowing for innervation and subsequent locomotion of smooth and skeletal muscle. These two types of neurons, motor and sensory, are coupled together to form nerves which originate directly from the spinal cord and brain. The autonomic nervous system (ANS) is a specialized part of the PNS which is responsible for reflex actions, motor movements triggered in response to stimuli irrespective of CNS input [1].

The neuron, the functional unit of the nervous system, contains two processes, a dendrite and an axon, which are connected to a cell body, as shown in Figure 2.
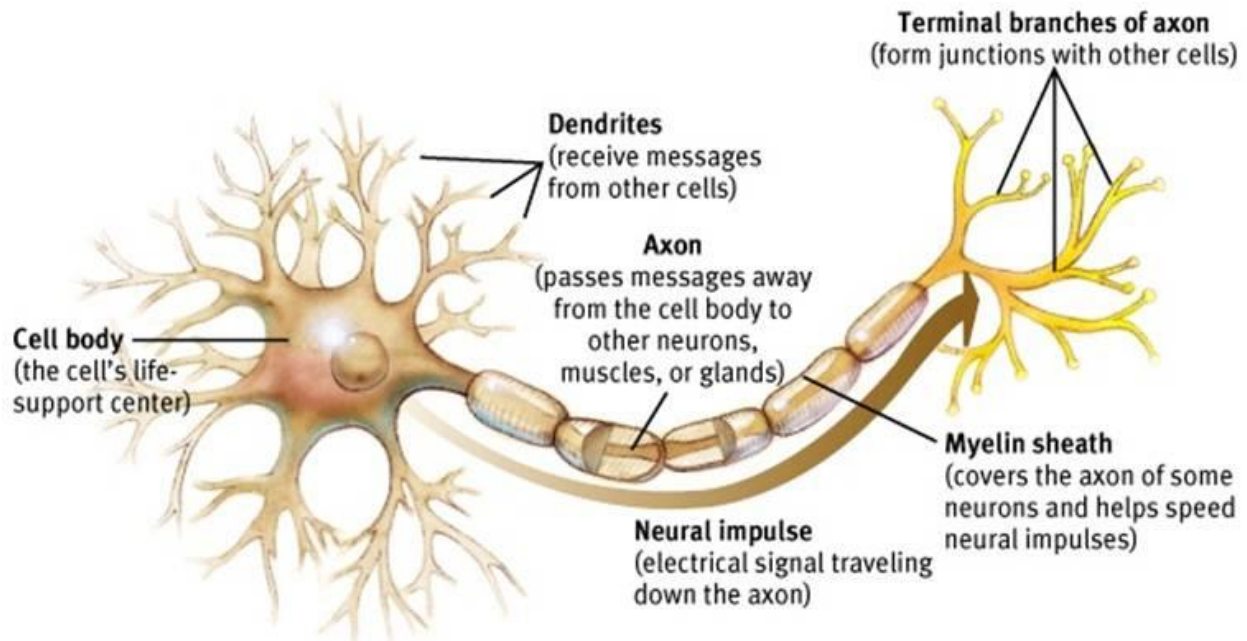
Figure 2: Anatomy of a neural cell body.

Axons terminate in a synapse, which is utilized in signal communication via the release of a neurotransmitter. This neurotransmitter binds to a receptor protein on the postsynaptic membrane of the receiving neuron, causing the opening of an ion channel. Negatively or positively charged ions flood into the cell via this open ion channel, causing respectively either depolarization or hyperpolarization of the membrane. Depolarization at a synapse evokes a

transient electro-chemical signal which travels down the dendrite and cell body by opening voltage-gated ion channels through depolarization of subsequent sections of the membrane[2]. Should this signal reach the axon hillock, identified in Figure 3, without a loss of significant amount of energy, an action potential will result, propagating towards the next synapse.

The conduction velocity of such a signal is, in general, directly related to myelination, axon diameter, and the distance between the nodes of Ranvier of the myelin sheath. A-type fibers, which are myelinated and larger (2-20 μm) have conduction velocities varying between 12-120 m/s. B-type fibers, myelinated fibers with smaller diameters (<3 μm) than those of A-type fibers have conduction velocities ranging from 3-15 m/s. C-type fibers, which are even smaller (0.3-1.3 μs) and unmyelinated, conduct only between 0.5-2.3 m/s [3]. In myelinated fibers, nodes of Ranvier increase conduction velocities by allowing saltatory conduction, a sort of leap of depolarization waves between nodes.

Electrical Neural Stimulation and Recording

The gold standard for neurophysiological study has traditionally been centered around electrical methods. An electrode is placed on or adjacent to a neuronal target and a small current is applied, depolarizing the membrane of the neuron and evoking an action potential. This signal then propagates down the axon towards additional synapses or a target tissue, causing a measurable response such as a motor action. An example of an electrical stimulation and response is shown in Figure 3. Electrical stimulation has been utilized in the stimulation of multiple tissue types, including skeletal muscle, cardiac muscle in pacemaking applications, and deep brain regions for the treatment of neuropathies such as Parkinson's disease and Tourette's syndrome.
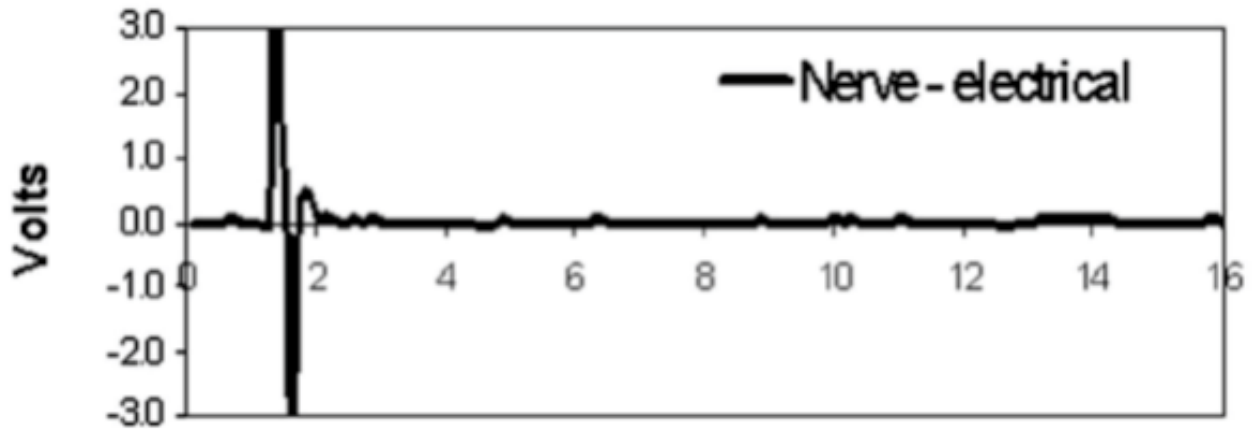
Figure 3: Electrical recording of single electrical stimulation. Horizontal axis units are milliseconds. [4]

Electrical stimulation has been used to map regions of the brain during surgery and has been utilized in the identification of hyperactive areas of the nervous system, allowing for the diagnosis of various disorders [2]. Furthermore, proper regulation of stimulation voltages and currents allows for stimulation conduction without tissue damage at the electrode-tissue interface[5]. Stimulation parameters have been well characterized, including current, voltage, pulse duration, repetition rate, and waveform, increasing the robustness of this technique.

Yet despite the efficaciousness of electrical stimulation which has earned it its place as the gold standard for clinical and research applications, this method is not without limitations. For instance, this method is limited regarding the stimulation of individual neurons. The existence of an electrical stimulation artifact makes the simultaneous stimulation and recording of adjacent neurological tissue difficult without the use of complex forward-masking methodologies [4,6]. The spread of current concomitant to the usage of extracellular electrodes leads to the oft undesirable stimulation of neurons adjacent to the target tissue. Currently the only means of addressing this difficulty are to either place an electrode very close to the neuron of interest, or to utilize an intracellular electrode [7]. The impalement of a neuron in this manner leads to the apoptosis of the

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cell upon electrode removal[8]. Due to these limitations in traditional electrophysiological techniques, which lead to a lack of spatial precision, a number of modalities have been developed to address them. Infrared neural stimulation methods are among them.

Infrared Neural Stimulation and its Current Applications

Infrared neural stimulation (INS), the use of pulsed infrared light to stimulate an action potential, has seen a growth in interest in recent years in the realm of neurophysiological study in both the central and peripheral nervous systems [4,9–11]. This increase in interest stems from a number of advantages inherent to INS. INS has a high degree of spatial acuity, allowing for the selective stimulation of a small number of neurological targets. Furthermore, depending on application, it is also a potential contact-free method of stimulation, requiring no physical contact between target tissue and the stimulation source. This overcomes one of the limitations present in other methods of stimulation, such as the necessity of physical contact and often intrusion of an electrode in order to selectively stimulate an individual neuron or axon, which oft introduces trauma to the tissue being studied. Another beneficial aspect of INS is the absence of a stimulation artifact. In many applications wherein electrophysiological recordings are performed for the observation of a response to a stimulus, there is a stimulation artifact, a presence of the electrical stimulus in the recording, that is unavoidable due to the fact that the stimulation modality occurs in the same domain as the recording technique. When recordings are made adjacent to a site of stimulation, the neurophysiological response is contaminated by the electrical field used for stimulation. This has prompted the development of numerous techniques for the reduction of this artifact. INS is not affected by these restrictions. This is because INS, as it is currently understood, functions through the introduction of a thermal gradient, rather than

through an electrical current [12–15]. As INS avoids these limitations, it may serve as a viable adjunct to electrical methods of neurophysiological study.

As an optical modality of neural stimulation, parallels may be drawn between INS and the field of optogenetics. Optogenetics is a neuromodulation technique that takes advantage of the introduction of exogenous light-sensitive proteins into target tissue, allowing for selective stimulation of only those tissues expressing these proteins when exposed to the proper wavelength of light[16]. Although optogenetics has seen tremendous success and utility in the field of neurophysiological study, its applications are limited by the need for exogenous factors, which confounds introduction into a clinical setting. INS does not require the use of exogenous factors or other modification of target tissues, thereby avoiding this limitation.

INS has seen use in a number of applications including, though not limited to, cochlear stimulation [17–19], somatosensory cortex stimulation [20], visual cortex stimulation in non-human primates [21], cardiomyocyte pacemaking [22], and stimulation of the peripheral nervous system [23]. There has also been ongoing study into its viability in the identification of damaged nerves during dorsal root rhizotomy procedures. However, questions remain extant about the viability of INS as a tool for neurophysiological assessment.

Motivation

INS has a proven robustness in neurophysiological applications, but there have been roadblocks impeding its introduction into the clinic. One challenge facing this clinical push is maintaining repeatability and constancy of stimulation within a safe window of stimulation. Variability has been observed in the threshold of radiant exposure needed to stimulate neural targets, which calls to question the issue of repeatability, with reproducibility of stimulation observed to be as low as

23% [24]. There are methods to address this, such as continuous monitoring of neural responses and adjustment of stimulation parameters as necessary, but these require a level of attention that is not feasible in a prolonged study and would not be acceptable in a clinical application. Furthermore, INS does an excellent job of stimulating small regions of interest, but clinical applications such as nerve monitoring require a more ubiquitous stimulation of tissue. Increasing laser power and/or spot size is a potential solution to both of these questions; if it can be done safely.

Increasing radiant exposures to levels above stimulation threshold includes a concomitant concern for thermal damage with higher energy pulses. There is threshold of safe radiant exposures beyond which damage is much more likely to occur. Comparing stimulation thresholds and safety thresholds yields a safety ratio, a metric of how much additional energy can be used without risk of damage. Wavelengths used in earlier studies had safety ratios on the order of 2:1 [25], which is insufficient for a clinical application. Improving this safety ratio is the primary means of facilitating the translation of INS into the clinical setting.

Hypothesis and Objectives

A majority of previous INS work has utilized the 2120 and 1875 nm wavelengths. While these studies have demonstrated that INS is capable of evoking neural responses, there has been an omnipresent question of the repeatability and safety of INS in generating these responses. Previous safety ratios between damage and stimulation have been on the order of 2:1, which is insufficient for a clinical setting. Repeatability has also been an issue, with stimulation thresholds varying within an animal on a temporal basis, leading to reproducibility below 25%. This leads to far too much ambiguity: the functionality of a nerve cannot be accurately assessed if there is uncertainty as to whether or not a proper stimulus has been applied to it. It must be noted, however, that in actual application all lasers produce a wavelength range, rather than a single individual wavelength. The previously utilized 1875 nm light is located on a slope for water absorption (Figure 4), the primary chromophore for INS; it is possible that variance in the actual wavelength generated by the laser leads to an increase or decrease in heat generation.
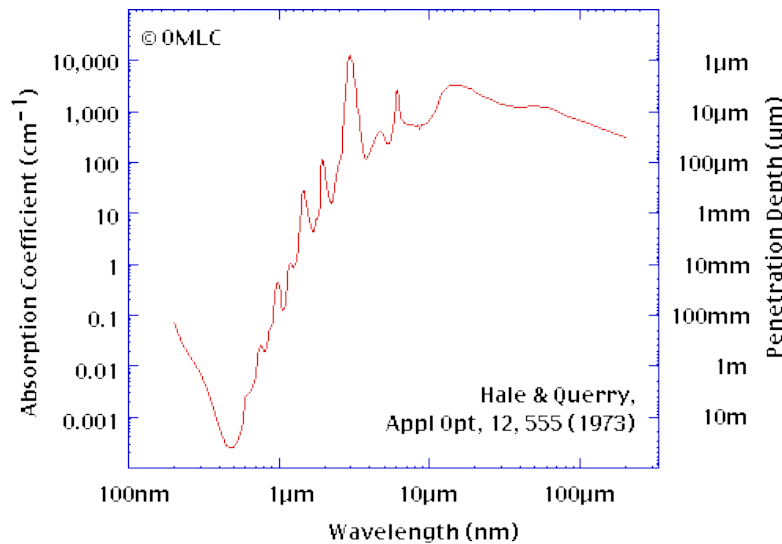

Figure 4: Light absorption properties of water.

By this assumption it may mean that a wavelength centered on a relative peak, wherein slight variance in wavelength does not lead to a large variance in absorption, will produce a more constant and reproducible stimulation. Light sources in the 1.4-1.5 µm range are also more readily available than 1.8-1.9 µm sources due to the telecom industry, meaning a more ubiquitous availability of sources of optical stimulus.

There are three main objectives to this work aimed at improving the performance of INS and demonstrating its clinical relevancy. The first objective is to determine if differing wavelengths of similar absorptive characteristics exhibit differences in stimulation and damage thresholds and resulting safety thresholds. If a difference is found, this would indicate a potential avenue of exploration for the improvement of INS safety and efficacy. The second objective is to determine if laser spot size and spatial beam profile has an effect on stimulation thresholds. This objective will elucidate whether or not larger spot sizes may be used for more ubiquitous stimulation, and if the energy may be more evenly distributed for a mitigation of damage risks. The final objective is to assess whether or not INS may serve as an adjunct to electrical methods in the clinic in the assessment of nerve damage. It is believed that electrical signal may be conducted through, or more accurately, around damaged nerves, while INS-evoked signals will not.

This thesis focuses on the improvement of INS safety ratios, and on demonstrating its clinical application and relevancy. The results from this thesis will guide future studies and ease the transition of INS towards its use as a diagnostic tool in the clinical setting.

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CHAPTER 2

INVESTIGATION OF 1450 NM INFRARED LIGHT FOR CLINICAL NERVE STIMULATION

Introduction

Neural stimulation, the process of instigating action potentials through an external energy source in central and peripheral neurons, is a technique of import and utility in myriad neuroscientific fields. From neurophysiological study and nerve mapping, to nerve monitoring and functional restoration, the ability to perturb neurological systems has led to a greater understanding of the arrangement of the central and peripheral nervous systems. This understanding has allowed for treatments that address functional disorders such as Parkinson's disease, Tourette's syndrome, and the restoration of hearing to name a few.

The gold standard for study and treatment in the nervous system is electrical stimulation; however, this method is limited in the stimulation of individual neurons. The existence of an electrical stimulation artifact makes the simultaneous stimulation and recording of adjacent neurological tissue difficult without the use of complex forward-masking methodologies[1,2]. The spread of current concomitant to the usage of extracellular electrodes leads to the oft undesirable stimulation of neurons adjacent to the target tissue. Currently the only means of addressing this difficulty are to either place an electrode very close to the neuron of interest, or to utilize an intracellular electrode[3]. However, electrical methods are not the only means of stimulating action potentials. Mechanical methods are regularly employed in applications of tactile stimulation, such as two-point discrimination tests [4]. Magnetic fields have been utilized as a non-invasive means of transcranial

stimulation of the brain via the electromagnetic induction of depolarizing electrical currents, although this method is limited in its spatial resolution [5,6]. Chemical means of neural stimulation have also been explored via the alteration of ion concentrations in *in vitro* experiments, though the delivery of chemicals of interest and maintenance of ion concentrations *in vivo* is an extant challenge. Yet none of these methods sufficiently address the challenge of spatial selectivity, requiring exploration of alternative means of stimulation. Optical methods of stimulation, which utilize light as a mode of stimulus, address the challenge of spatial selectivity.

Optical stimulation improves spatial selectivity, a necessary feature in the stimulation of discrete neurological targets, as it only perturbs those regions of tissue that are illuminated. Optical methods include neurotransmitter uncaging, optogenetics, and infrared stimulation (INS). Neurotransmitter uncaging utilizes the activation of exogenous neurotransmitters with two-photon illumination methods, and may be seen as unification of light and chemically mediated modes of stimulation [7]. Optogenetics takes advantage of the selective upregulation of light-sensitive channels such as channelrhodopsins using adeno-associated viruses, and allows for specific site activation based on protein uptake [8]. However, both of these methods require the introduction of exogenous factors, which impedes the path to clinical translation.

INS is a thermally mediated method of optical stimulation which requires no prior preparation of target tissue outside of exposure. It has been shown to perturb target tissue through a temperature gradient attributed to an alteration of membrane capacitance [9,10]. INS utilizes pulsed or continuous wave infrared laser light in the stimulation of neural targets, and has been shown viable in the stimulation of cardiomyocytes [11], hair cells of the cochlea [12,13,14], the somatosensory and visual cortex [15,16], the sciatic nerve [1,2,17], the cavernous nerve [18,19], and the facial nerve of the peripheral nervous system [20]. This diversity of applications, coupled with its spatial selectivity and absence of

exogenous factors, demonstrate the potential utility of INS in not only neurophysiological study, but in clinical applications as well. However, certain questions and challenges impede this clinical translation.

One challenge facing this clinical push is maintaining repeatability and constancy of stimulation within a safe window of stimulation. Variability has been observed in the threshold of radiant exposure needed to stimulate neural targets, which calls to question the issue of repeatability, with reproducibility of stimulation observed to be as low as 23% [21]. There are methods to address this, such as continuous monitoring of neural responses and adjustment of stimulation parameters as necessary, but these require a level of attention that is not feasible in a prolonged study and would not be acceptable in a clinical application. Furthermore, although INS does an excellent job of stimulating small regions of interest, clinical applications such as nerve monitoring require a more ubiquitous stimulation of tissue; it is unknown if a change in beam size or shape will affect repeatability. Increasing laser power is a potential solution to issue of repeatability; if it can be done safely.

Increasing radiant exposures to levels above stimulation threshold includes a concomitant concern for thermal damage with higher energy pulses. There is threshold of safe radiant exposures beyond which damage is much more likely to occur. Comparing stimulation thresholds and safety thresholds yields a safety ratio, a metric of how much additional energy can be used without risk of damage. Wavelengths used in earlier studies had safety ratios on the order of 2:1[2], which is not ideal for a clinical application. This prompted the investigation of a new wavelength, 1450 nm, for the purposes of improving this safety ratio of stimulation and ultimately repeatability. The purpose of choosing this wavelength is its similar absorptive properties in water when compared to 1875 nm light.

We investigated other factors in addition to wavelength, with the aim of improving INS safety thresholds and repeatability for the purpose of clinical translation. We addressed whether or not a change in radiant exposure is necessary to reliably stimulate a neurological target when a laser spot size is varied in the interest of increasing or decreasing the region of stimulation. This is of importance when considering the variations in size and locations of neurological targets. We also investigated beam shape and the question as to whether or not a change in the distribution of energy across a laser spot will alter the thresholds necessary for activation. Previous research utilized a Gaussian beam profile, which creates a center region of increased heat generation. A flat-top beam profile features a uniform light distribution as compared to the traditional Gaussian profile. If thresholds between the two profiles are identical, the more even distribution of energy of the flat-top profile could mean a mitigation of thermal damage risks.

Finally we performed a study utilizing clinical hardware and software based on observations in the outlined experiments, in order to further assess the usefulness of INS in a clinical application. We made a comparison of stimulation modalities, INS and electrical, in healthy and damaged nerves, with recordings taken distal to the site of injury using the NIM 2.0 nerve monitoring system. The differing modes of stimulation and absence of an artifact in optical stimulation revealed INS to be a potential adjunct to electrical in the identification of damaged nerves; a necessary task for nerve monitoring.

The findings of this research illuminate a number of means of improving stimulation repeatability and increasing safety margins. Here we demonstrate that 1450 nm light enables safe and repeatable stimulation at multiple nerve locations, something not feasible with wavelengths used in earlier research. By demonstrating safety and repeatability, we have ultimately facilitated the clinical translation of INS.

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Methods

All experiments were conducted at the Vanderbilt University W.M. Keck Free Electron Laser Center and Vanderbilt Biomedical Optics Laboratory following approval by the Institution of Animal Care and Use Committee.

Rat preparation

In vivo sciatic nerve experiments were performed using male Sprague-Dawley rats (n = 33) (250-350 g), purchased from Charles River labs. Animals received anesthetization with continuously inhaled isoflurane. A rectal probe and heating pad (catalog# 40-90-8, FHC, Bowdoin, ME) maintained a target body temperature for the rat of 36-37° C for the duration of experiments. Animals rested on a polycarbonate platform and fitted with a nose cone for continual isoflurane anesthesia; front limbs were taped into place to secure the animal's position. Both hind limbs were shaved, and the dorsal surface of the foot was taped to the edge of the platform. The length of the hind paw was kept perpendicular to the working surface. An incision was made in the skin starting above the Achilles tendon and proceeding towards the vertebral column. The skin was separated from the underlying tissue to expose the biceps femoris. The biceps femoris was then cut and divided proximal to the Achilles tendon, allowing identification of the sciatic nerve. The incision continued towards the vertebral column; upon completion, towel clamps separated the biceps femoris and keep the main trunk of the sciatic nerve exposed (Figure 1). The epineurium was then dissected away from the sciatic nerve.
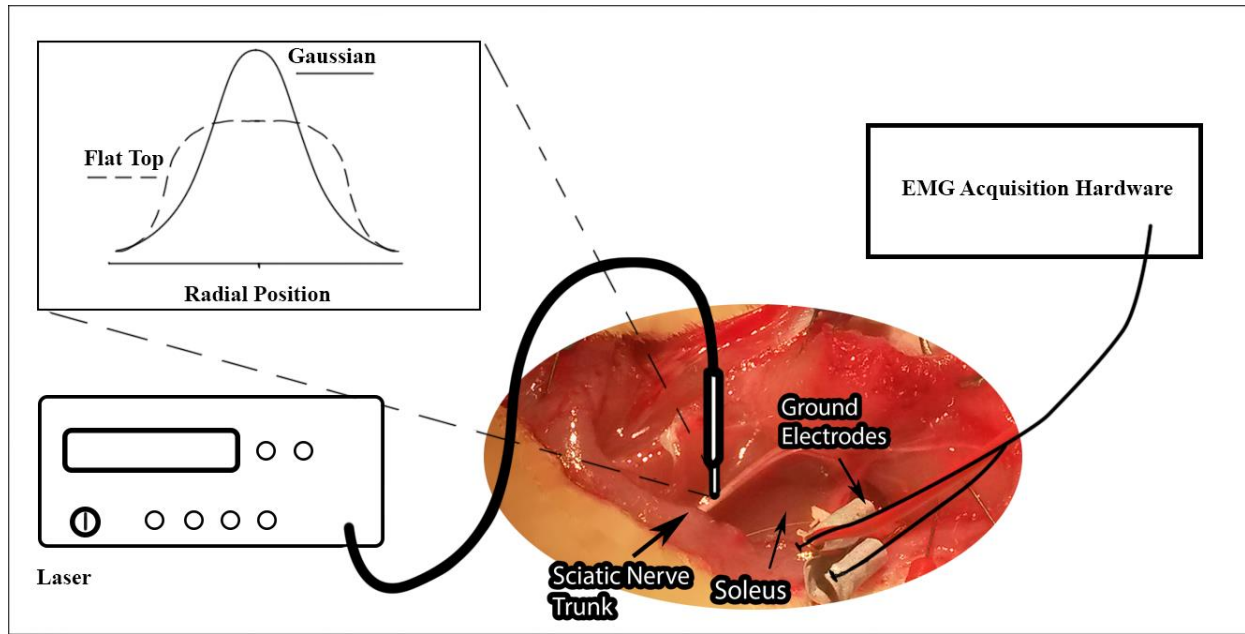
Figure 1: Schematic of surgical preparation, including laser, fiber positioning over sciatic nerve, and electrode placement in soleus muscle. Inset shows Gaussian and flat-top laser beam profiles utilized in study.

Optical stimulation

Silver electrodes were inserted into the soleus muscle for the recording of EMG signals, using the DigiData 1440A Data Acquisition (Molecular Devices) system and Axograph software. Input signals were amplified by 1000, and filtered through a 100-1000 Hz band pass filter.

Optical stimulation was delivered using two types of fiber optic probes. Polished bare fibers (Ocean Optics, NA = 2.2) provided a Gaussian beam profile, with spot size adjusted by distance of the fiber tip to the site of stimulation; spot sizes were measured using the knife-edge method[22], which provided spot sizes accurate within ±50 µm. A custom-made fiber optic probe, manufactured to generate a 1 mm spot size for the wavelengths utilized at distances up to 5 mm from the probe tip, provided a flat-top beam profile. The probes were connected to one of two tunable pulsed diode lasers of different wavelengths (Lockheed Martin Aculight), then positioned and held in place using

a micromanipulator (World Precision Instruments) for these experiments. Two wavelengths ($\lambda_1 =$ 1450 nm $\lambda_2 = 1875$ nm) were utilized in this study. Repetition rate was kept constant at 2 Hz; diode power and pulse widths were adjusted to vary radiant exposure during experiments.

For each experimental procedure, animal sciatic nerves received stimulation in 10 second trains for a total of 20 pulses per train (2 Hz), and EMG responses were recorded. Stimulation threshold was defined as the radiant exposure at which 50% activation of a targeted nerve occurred. Stimulation was said to have occurred when a visual change (~0.2 mV) was observed in the baseline signal of the recording electrodes.

Wavelength Comparison

Animal studies (n = 6) to discern general differences between 1450 nm and 1875 nm light were conducted to establish an optimal wavelength for repeatable and safe laser excitation of neural tissue. Identical trials were performed for each wavelength, beginning with an assessment of the stimulation threshold for an 800 μm Gaussian spot size centered on tibial branch of the sciatic nerve. Additional experiments included a spot size comparison (n = 6) within and across wavelengths for 500, 1000, and 1500 μm spot sizes. Within these experiments, the effect of beam profile was also assessed at the 1000 μm spot size diameter. The experimental protocol followed the methods described previously, with 10 second trains of 20 pulses employed and EMG responses measured using AxoGraph software. Comparisons were made using unpaired t-test analyses.

Histological damage study

Animals (n = 12) were prepared via the surgical protocol described above. A 400 μm optical fiber (NA = 0.22; Ocean Optics) delivered optical stimulation at a distance of 1.5 mm from the sciatic nerve, creating a spot size of approximately 500 μm. Spot size was measured prior to experiments.

The two tunable pulsed diode lasers described in the previous experiment (λ_1 = 1450 nm, λ_2 = 1875 nm) were used in this study. A train of 20 pulses was triggered using AxoGraph software and a Digidata 1440A Data Acquisition system at a repetition rate of 2 Hz. Radiant exposures of approximately 6.00 J/cm^2 served as positive controls. A negative control was created by positioning the fiber similarly to other trials, but omitting the step of irradiation. Three additional spots varied between the following radiant exposure values: one times 1875 nm threshold, two times 1875 nm threshold, and three times 1875 nm threshold. These three radiant exposures were used for both 1450 nm and 1875 nm trials. There were five trial spots in total proceeding axially along each nerve, with a distance of at least 1 mm between each.

Positive control spots were marked with black marking dye (Thermo Scientific), negative control spots were marked with yellow marking dye (Thermo Scientific), and all other irradiated spots were marked with blue tissue stain (Delasco). After stimulation, nerves were immediately excised from the hind leg and placed in 4% paraformaldehyde for fixation. Nerves fixed for a period of at least 48 hours and were then taken to a histologist for paraffin embedding, slicing, and toluidine blue staining. Toluidine blue was chosen for its functionality as a myelin stain.

Nerve monitoring study

Animals (n=9) were prepared via the surgical preparation described above, with omission of epineurium removal. Muscular action potentials were measured using the NIM 2.0 Nerve Monitoring System (Medtronic) in three locations: the gastrocnemius, the soleus, and the anterior tibialis. Nerves received optical stimulation from the 1.0 mm flat-top probe from Aculight, at radiant exposures ranging between 1.4-1.6 J/cm^2, and monophasic electrical pulses using the NIM 2.0 probe at amperages ranging between 0.03-0.10 mA. Nerves were stimulated at 2 Hz optically and 4 Hz electrically. Stimulations were repeated on the nerve after a 15 minute period to confirm continued

functionality, and then the nerve was cauterized. Following cauterization, the stimulation protocol described above was employed, and signals were recorded distal to the site of injury.

Results

Effect of wavelength on stimulus response

Stimulation trains were introduced in the rat sciatic nerve using 1450 nm and 1875 nm light. The percentage of stimuli that instigated muscular action potentials were compared to a given radiant exposure per animal (Figure 2).
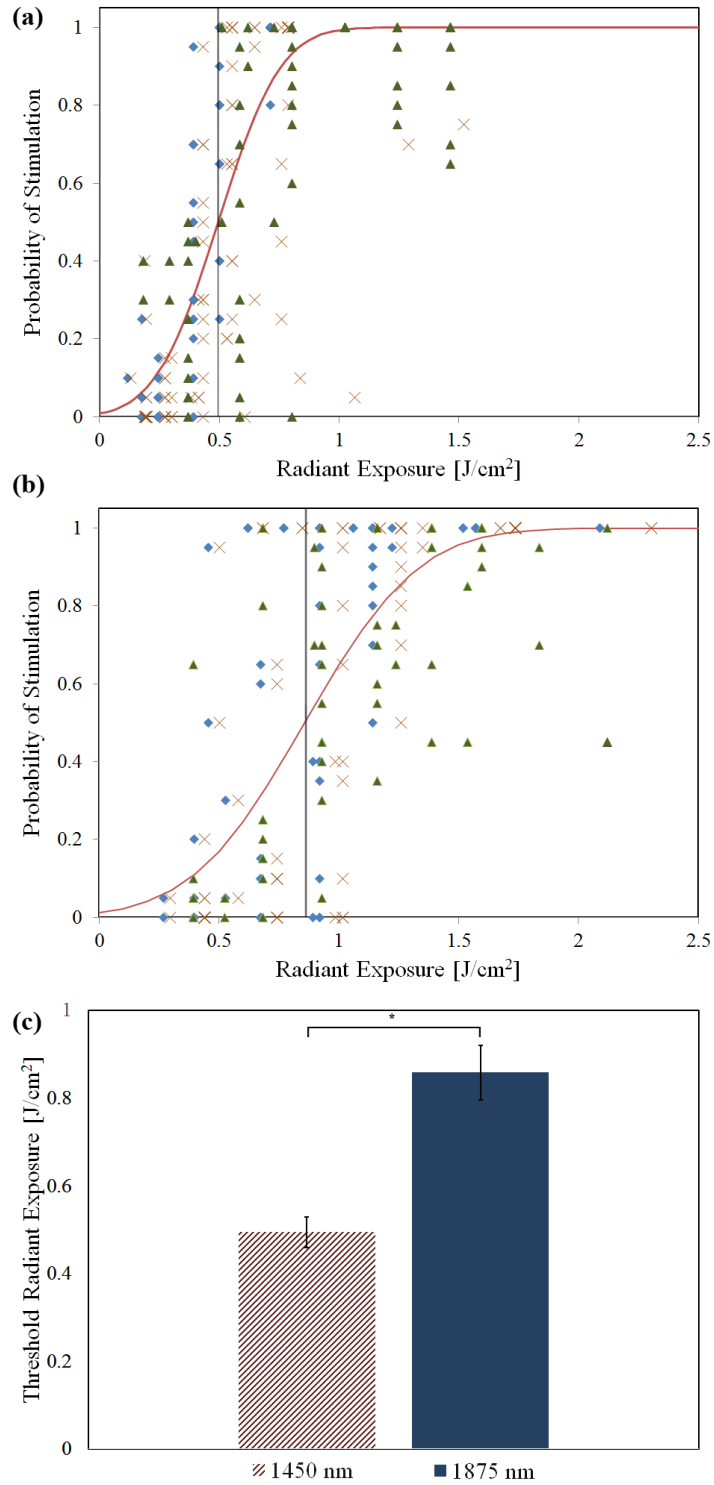
Figure 2: Stimulation Threshold for a) 1450 nm light b) 1875 nm light. Different markers indicate different animals. Rate of activation refers to the number of measurable responses (electrophysiological) per total stimulations. Repetition rate: 2 Hz. Spot size diameter 800 μm. c) Significant difference was observed between wavelengths, with 1450 requiring lower radiant exposure. Gaussian beam profiles were employed. * indicates p < 0.05.

It was observed that between animals the stimulation threshold for 1450 nm light demonstrated less variation between animals than 1875 nm light in the sciatic nerve. There is an intermediate range where a given radiant exposure's probability of instigating an action potential varies between 0 and 1, that was present in 5 of the 6 animals. The 1450 nm light exhibited a statistically significant lower stimulation threshold ($0.493 \pm .084$ J/cm^2) in comparison to 1875 nm (0.858 ± 0.084 J/cm^2) (n = 6 animals, $p < 0.05$) (Table 1) (Figure 3).

Spot Size [μm]	1450 nm [J/cm^2]	1875 nm [J/cm^2]
500	$0.717 \pm .067$	0.652 ± 0.045
800	$0.493 \pm .084$	0.858 ± 0.084
1000	$0.472 \pm .110$	0.694 ± 0.097
1000 (Flat Top)	0.589 ± 0.030	0.772 ± 0.054
1500	$0.563 \pm .034$	0.995 ± 0.083

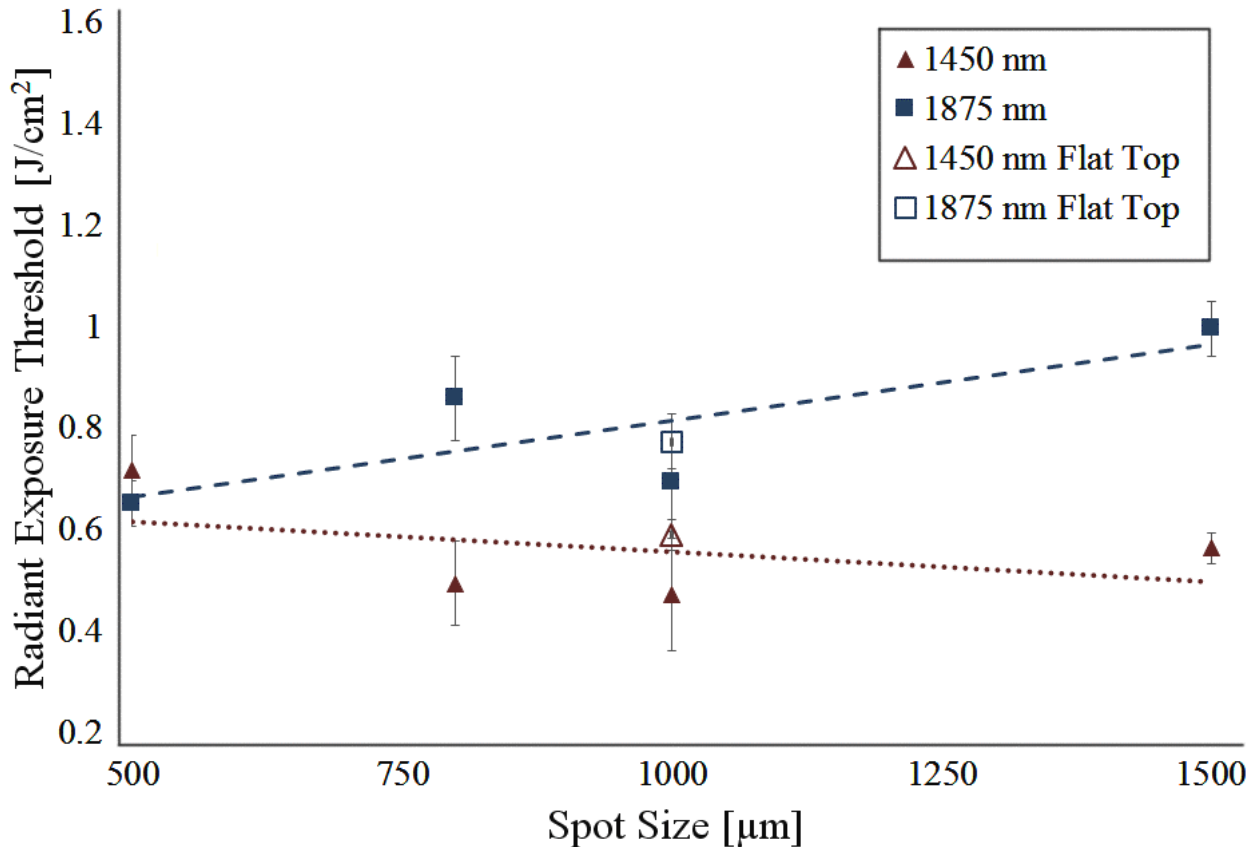Table 1: Threshold radiant exposures per wavelength and spot size.

Figure 3: Stimulation thresholds between different spot sizes and wavelengths. The inset contains a comparison between wavelengths for the 1000 μm flat-top probe. * indicates *p < 0.05*.

Effect of Beam Profile and Spot Size on Stimulation Threshold

Gaussian and flat-top beam profiles were compared in order to assess the effect of energy distribution in a laser spot. Comparisons between spot sizes (500, 800, 1000, and 1500 μm diameters), wavelengths (1450 and 1875 nm), and beam profile appear in Figure 3. Threshold radiant exposures according to spot size and beam profile are given in Table 1. Although statistically significant differences (p < 0.05) in thresholds were observed between 500 μm and 1500 μm Gaussian spot sizes within wavelengths, there was no prominent trend observed when comparing 1450 nm and 1875 nm light. An increase of stimulation threshold was concomitant with increasing

spot size for 1875 nm light, but this pattern of behavior was not observed for 1450 nm light. There was no statistically significant difference in stimulation threshold between flat-top and Gaussian beam profiles of equal diameter for either 1450 nm or 1875 nm light (p>0.05).

Histology

A histological analysis was performed on excised nerves following acute stimulation within the main trunk of the sciatic nerve for the purpose of assessing and quantifying damage within neural tissue following irradiation. Comparisons between a negative control, a positive control, and approximately three times the 1875 nm threshold are shown in Figure 4.
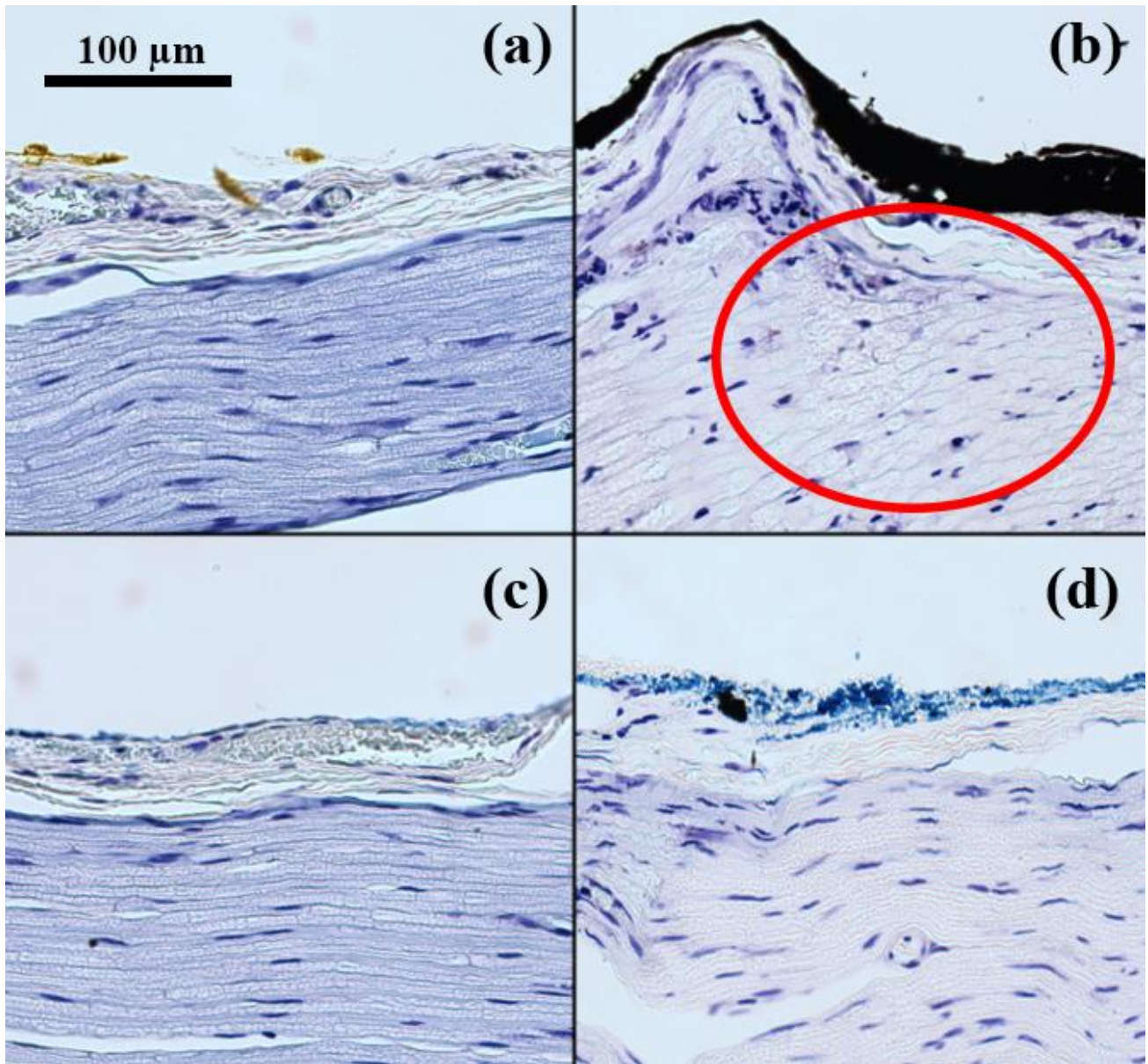
Figure 4: Toluidine-blue stained irradiated nerve slices. Histological images taken at 60x (a) Negative control, no stimulation, (b) positive control: 6 J/cm² 1450 nm light, (c) 3.08 J/cm² irradiation at 1450 nm(d) 3.15 J/cm² irradiation at 1875 nm. Ellipse indicates region of damage.

Damaging levels of radiant exposure (6 J/cm^2) produced disturbances in the myelin of the peripheral nerve, with the greatest disruptions present in the center of the irradiated area; axonal striations were absent in the positive control (Figure 4b). Radiant exposures up to three times that of 1875 nm light threshold did not produce observable damage for 1450 nm or 1875 nm light. In Figure 4c and 4d, the integrity of the myelin is preserved, without the rupturing extant in Figure 4b.

Nerve monitoring study

Both optical and electrical stimuli were able to perturb a measurable response in the gastrocnemius, soleus, and anterior tibialis muscles as shown in the comparison given in Figure 5. Electrically evoked responses were greater than those evoked optically by an order of ten within healthy nerves. After nerves were cauterized, only electrical stimulation evoked a measurable signal distal to the site of injury, due to current spread.
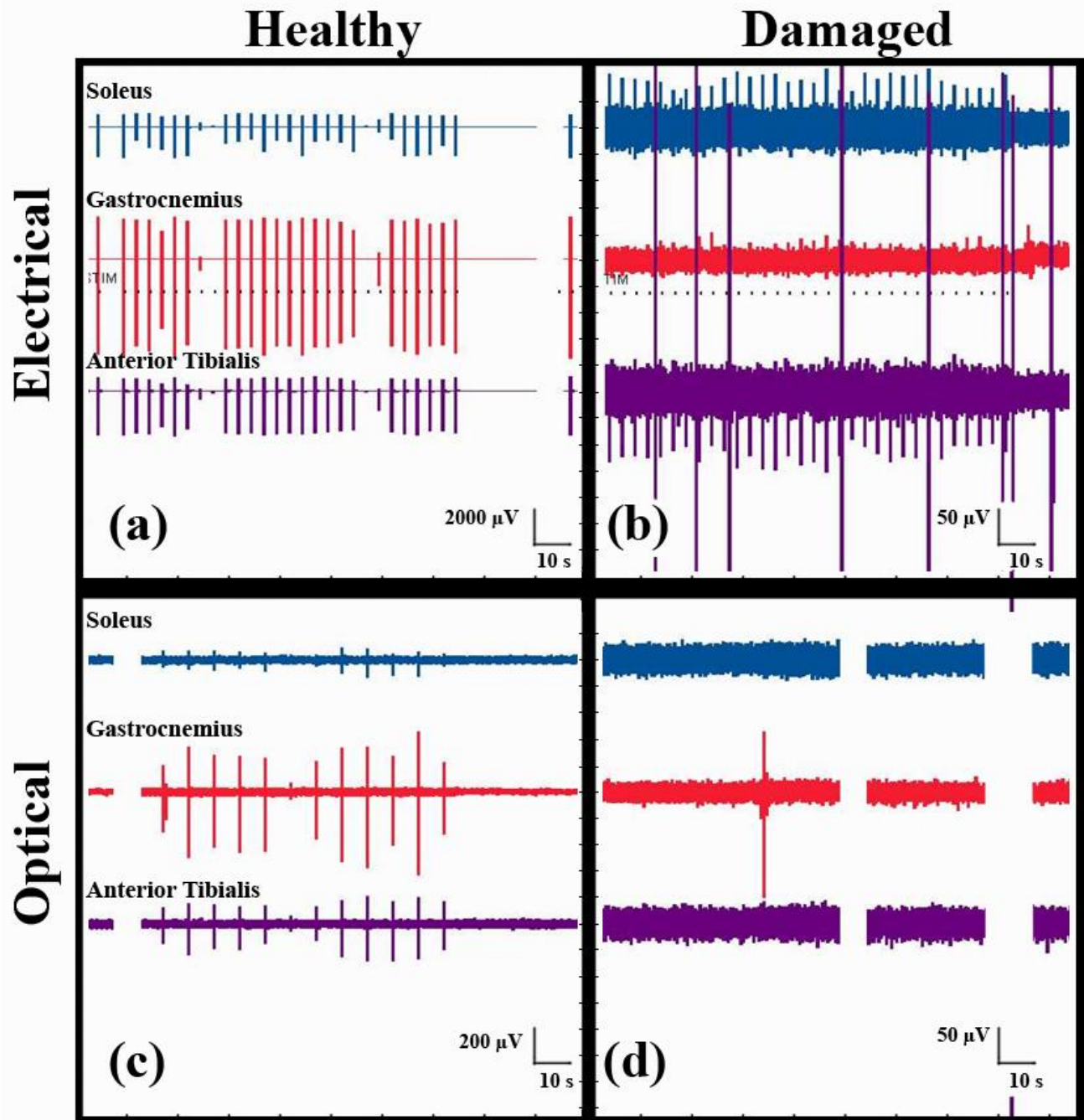
Figure 5: Response comparison before and after damage, all four horizontal axes: 10 s. (a) vertical axis range: 2000 µV, (b) vertical axis range: 50 µV, (c) vertical axis range: 200 µV, (d) vertical axis range: 50 µV.

Discussion

Infrared light has a track record of success in the stimulation of numerous physiological targets, proving a spatially selective means of perturbing action potentials without the need for exogenous factors. It is readily paired with multiple means of neural monitoring such as electrical recordings, calcium staining and fluorescence imaging [15], intrinsic imaging [23], and fMRI [24], a difficult avenue for electrical stimulation. However, although INS has been refined as a tool of neurophysiological study, there have been challenges that required address, including a difficulty in maintaining repeatability in experiments while maintaining radiant exposure values within a safe window. Previously explored methods addressing this include hybrid methods of stimulation which combine below-threshold optical and electrical stimuli which, when combined, cause activation of a neurological target [17,25]. Yet even within these methods a temporal effect has been observed while using 1875 nm light, requiring continual monitoring and adjustment of laser parameters. This hinders repeatability and is an inconvenience that eliminates clinical utility. Increasing laser power relative to threshold has been a hitherto unavailable option. This paper accomplishes this goal through the parameterization and application of a previously unexplored wavelength of light in the field of optical stimulation.

Here we provide another means of maintaining safe radiant exposure levels with increased repeatability through the use of a new wavelength of light: 1450 nm. We found statistically significant reductions in the threshold radiant exposure when using 1450 nm light for all but one spot parameter as compared to 1875 nm. Our histological findings did not demonstrate a reduction in damage thresholds, indicating an increase in the margin of safety through the use of this wavelength of light. This work also highlights the efficacy of flat-top beam profiles as compared to the previously utilized Gaussian profile, showing no statistically significant difference between the two. Spot size was also investigated and found to have a minimal effect on threshold radiant exposure,

meaning a more ubiquitous irradiation of target tissue is viable without increasing radiant exposure to damaging levels. This work was supported through the demonstration of infrared light's utility in clinical applications in a nerve monitoring scenario, wherein INS showed a greater propensity for the identification of damaged nerves.

The viability of 1450 nm light in INS applications is evident when performance is compared to that of 1875 nm light. Stimulation thresholds are reduced in a statistically significant degree for 1450 nm light, with repeatable stimulations more easily attained at lower values of radiant exposure and identical repetition rates. This reduction in threshold means a mitigation in the risk of thermal damage. It should be noted that 1450 nm and 1875 nm light have similar absorptive properties in water [26]. However, 1450 nm light is on a relative peak on the water absorption curve, while 1875 nm light is on a prominent slope. This fact, coupled with the reality of the broadband nature of the diode laser light, may account for the increased repeatability demonstrated by 1450 nm light. Although there was an apparent direct relationship between spot size and stimulation threshold within the 1875 nm wavelength, there was no such relationship observed for 1450 nm light. The question as to why a potential relationship between spot size and threshold appeared for the 1875 nm light may be addressed by the positioning of the fiber itself, and the type and percentage of axonal tissue irradiated as the spot size was increased. The sciatic nerve is well-organized, with sensory and motor axons occupying different areas of it [27]. It is possible that as spot size was adjusted, the encompassed area did not fully include motor axons, the tissue type responsible for the magnitude of measured responses.

The flat-top beam profile exhibited no significant difference in stimulation threshold for a 1000 μm size when compared to the Gaussian profile. This is in contrast to previous literature in which a flat-top profile required lower radiant exposure [28], though this could be attributed to differences in

experimental protocol. There was a decrease in stimulation threshold variance for the flat-top beam profile as compared to the Gaussian profile, which may be attributed to the collimation of the light emitted from the probe. The beam is collimated to a 1 mm diameter spot size at distances up to 5 mm away, reducing the variation in spot size that occurs concomitantly with variations in probe tip distance from the site of stimulation with bare fibers. A Gaussian profile does not have an even distribution of energy; rather there is a maximum of energy deposition located within the center of the laser spot. Subsequently, there appears to be an increased risk of thermal damage at higher values of radiant exposure centered at the site of irradiation. A majority of damage was centered within 100 μm for a 500 μm spot size. The flat-top profile's distribution of energy is much more uniform as compared to that of the Gaussian profile. The elimination of a hot spot could lead to a reduction in the risk of thermal damage at similar values of radiant exposure for a given wavelength, although this would be best determined by additional histological analysis. The functionality of the flat-top probe demonstrated in the comparison and nerve monitoring studies agrees with results presented by Tozburun et al., wherein the rat cavernous nerve was stimulated successfully and efficaciously with a 1 mm spot size flat-top beam[28]. This finding presents an added clinical viability in the elimination of spot size variation when the probe is held by a practicing physician.

The histological analysis performed revealed no acute thermal damage for either 1450 nm or 1875 nm light at radiant exposures up to 3.1 J/cm^2, the maximum value of radiant exposure used below the positive control. This is an encouraging result in particular for the 1450 nm light, representing the potential for safe stimulation at greater than five times its threshold for stimulation. This is a marked improvement over the 2:1 safety ratio observed in earlier work[2]. A 5:1 safety ratio allows for the use of larger radiant exposures to safely stimulate neural tissue, increasing the repeatability during the course of a procedure: an important requirement for clinical application, although it is

important to note that nerves were excised and fixed within 30 minutes after irradiation, and any chronic effects of infrared stimulation were not evaluated within the scope of this work.

The histological results and those detailed prior spurred the exploration of INS as a nerve monitoring modality in this work. Clinical monitoring hardware and software was utilized in the nerve monitoring study, and no additional preparation of the nerve was performed outside of exposure. The purpose of these conditions was to represent the clinical setting as faithfully as possible. It was theorized that a larger spot size could be used for a more ubiquitous irradiation of the sciatic nerve with no significant increase in radiant exposure for the course of the nerve monitoring experiments. This assumption was based on the results of the previously discussed spot size experiments. The flat-top profile was selected for the nerve monitoring experiments due to its comparable performance to the Gaussian profile, and the collimated nature of the probe. The combination of the large spot size and beam profile enabled the uniform irradiation and stimulation of multiple motor axons, evoking muscular action potentials in the gastrocnemius, soleus, and anterior tibialis. Electric stimulation perturbed responses approximately one order of magnitude higher than that of INS, which can be attributed to the spread of current through the nerve. It is believed to be this current spread which also produced a measurable signal distal to sites of neural damage, a signal which was absent when INS was applied. Electrical stimulation produced a false positive for functionality in a damaged nerve whereas INS did not. This is due to the nature by which INS evokes action potentials.

The mechanism of INS is believed to be a temperature-rate dependent membrane capacitance change at the site of irradiation. Shapiro et al. demonstrated this phenomenon through computational modeling and irradiation of protein-free lipid bilayers [9]. Liu et al. demonstrated similar mechanisms through rapid heat shocks of cardiomyocytes[10]. A capacitance change is not the only theory that exists to explain the mechanism of INS, however. Additional theories suggest the activation of heat-

sensitive channels such as the TRPV1 species[17], or the alteration of calcium dynamics in organelles such as the mitochondria and endoplasmic reticulum[29]. It is possible that the mechanism of INS is a gestalt of all of these phenomena. What is known is that this mode of stimulation is highly localized, as demonstrated in previous electrophysiological and histological study of the cochlea[13,14], and in imaging studies of the cortex[15,23]. Only action potentials conduct along a nerve with INS, as opposed to the addition of current that give rise to the stimulation artifact seen with electrical methods.

We have evaluated a new wavelength of light for the efficient and repeatable stimulation of neurons, and we've further established INS parameters regarding the threshold of stimulation. The results of this work establish 1450 nm light as a more efficacious wavelength as compared to the previously utilized 1875 nm light, with lower stimulation thresholds and increased repeatability. Furthermore we've demonstrated that the flat-top beam profile is comparable in performance to the Gaussian beam profile, which yields another means of reducing the risk of non-uniform thermal damage during experimental procedures. Through these results we've demonstrated a safer and more efficacious means of performing INS, increasing its utility to researchers and its ease of transition to the clinical setting. There it can increase the quality of care through the identification and assessment of nerve health.

Acknowledgements

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CHAPTER III

FUTURE DIRECTIONS

We have evaluated a new wavelength of light for the efficient and repeatable stimulation of neurons, and we've further established INS parameters regarding the threshold of stimulation. The results of this work establish 1450 nm light as a more efficacious wavelength as compared to the previously utilized 1875 nm light, with lower stimulation thresholds and increased repeatability. Furthermore we've demonstrated that the flat-top beam profile is comparable in performance to the Gaussian beam profile, which yields another means of reducing the risk of non-uniform thermal damage during experimental procedures. Through these results we've demonstrated a safer and more efficacious means of performing INS, increasing its utility to researchers and its ease of transition to the clinical setting. There it can increase the quality of care through the identification and assessment of nerve health.

However, addressing one set of questions leads to additional ones. The efficaciousness of 1450 nm light has been proven, and it is theorized that it benefits from its location a relative peak of absorption. It is possible that other wavelengths centered on similar peaks might exhibit a similar constancy in performance, but there is the question about the coefficient of absorption itself, particularly in terms of penetration depth. It is worth exploring whether or not wavelengths with a greater penetration depth would allow for stimulation of deeper regions of interest. One potential means of exploring this would be an application similar to two photon uncaging, wherein two light paths are set to converge on a region of interest, combining to activate an exogenous neurotransmitter [1]. Using INS, perhaps a similar effect could be achieved without the use of an exogenous substance.

Another question exists regarding the long-term effects of INS. The histological assays

performed in this study did not account for chronic usage of INS, and a study exploring the

effects of such an application would provide valuable information about the safety and efficacy

of INS.

The move towards clinical applications is necessarily gated by the completion of the

aforementioned studies; however, this does not impede exploration of additional utilizations of

INS. The spatial resolution of INS has already been shown to be efficacious in the stimulation of

the cochlea [2–4]. This spatial resolution may further serve in the development of prostheses which

restore not only the form and motility of a lost limb, but encode tactile and proprioceptive

sensory information as well. Previous work has revealed what appears to be an inhibitory effect

on neural signal conduction, which has significant implications for treatment of neuropathy[5].

INS can potentially serve as a treatment for chronic pain, spastic paraplegia, and other conditions

in which quiescence of nerves is required. Of course, elucidating and differentiating the

phenomena by which INS either activates or inhibits neural activity warrants attention.

We have observed that 1450 nm light can stimulate the peripheral nervous system reliably, so it

is worth assessing its utility in the central nervous system (CNS). In prior work 1875 nm was

used to activate CNS tissue in both *in vitro*[6] and *in vivo*[7–9] applications. The use of 1450 nm may

facilitate an INS means of cortical mapping, or even in treatments of neuropathies currently

addressed by electrical methods such as deep brain stimulation (DBS). The latter comes with the

caveats that the amount of light delivered, the metric by which stimulation scales, is limited by

the geometry of the fiber that transports it. Development of novel geometries may be necessary

to facilitate optimal light delivery while mitigating the trauma of introducing the fiber into

cortical tissue; such considerations and developed have already been made for optogenetic

applications [10]. The formation of glial scar tissue has been shown to impede conduction of electrical stimuli, and this is a relevant issue for optical stimuli as well [11–14]. This potential impedance should be the subject of rigorous study to determine the efficacy of INS in DBS applications.

The limitations of INS should be considered in the planning of future work, but by no means should its potential be dismissed. This work demonstrates that INS can be done safely and reliably, and that it provides a unique utility absent from other methods of neurological stimulation. The possibilities for INS are limited only by the imagination.

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