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DESIGNING A PLATFORM TO DETECT PERIPHERAL NEUROPATHY WITH MICRONEEDLE ELECTRODES AND NEUROPHYSIOLOGY

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biomedical Engineering)

The Honors College

University of Maine

May 2020

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ABSTRACT

The growing obesity pandemic has caused diabetes to become one of the world's leading health concerns. Diabetic individuals often suffer from peripheral neuropathy, which is nerve death that typically starts at the extremities and moves from the skin inward to deeper tissues. This nerve death causes painful symptoms including tingling, stinging, numbness, and others. Current methods to diagnose peripheral neuropathy by measuring nerve function are invasive and painful since they target large axons of the legs; however, by the time the neuropathy reaches the diagnosable axons, it is often too late for intervention. Although no cures for neuropathy are established, aside from analgesics for pain, there are options for mitigating worsening of the disease if diagnosed early enough (for example, glucose control for diabetic neuropathy). There is a critical need for early detection and diagnosis of peripheral neuropathy as obesity and diabetes continue to plague the world. Microneedle fabrication is a growing research area, especially in transdermal drug delivery due to their minimal invasive, pain-free application. The objective of this project is to design a platform using electrically conductive microneedles for early detection of peripheral neuropathy. Using these needles, neurophysiology can be employed to record electrical signal just below the skin to determine the integrity of the nerves to track neuropathy progression. Thus far, prototypes of the device have been tested on mice to establish protocols and understand the hardware and software, with the goal of eventually developing a usable prototype for a longitudinal study of diseased mice and human clinical studies.

ACKNOWLEDGEMENTS

My sincerest thanks to my Honors committee for sticking with me as I went through an unconventional Honors process. My advisors and others in the lab have been so supportive through everything the last couple of years.

I would also like to thank my family and friends for encouraging and supporting me through whatever life threw at me. My mother and father are my rock, and I wouldn't be where I am without them.

Other thanks to people who contributed and helped with this work:

Magdalena Blaszkiewicz- Ph.D., Mentor

Nuri Emanetoglu-Electrical Engineer, Collaborator

Julia Towne-Array Design and Development Contributor, REU Student 2018

Brooke Villinski-Recording and Overall Needle Array Development, REU Student 2019

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PROBLEM STATEMENT AND PROJECT GOALS

Diabetes is one of the most prevalent diseases across the world, and peripheral neuropathy is a common complication of such. The motivation behind this platform is to ultimately be able to diagnose this nerve death sooner than it currently is done in a minimally invasive and pain free method. There were several goals of this thesis to get the platform started and moving towards a direction to ultimately better the standard of care. The overarching aims of the thesis are as follows:

1. Develop protocols for both the hardware and software obtained to take electrical recordings in mice.
2. Use well-established nerve models to test and troubleshoot the hardware.
3. Reduce noise of recordings and understand the custom needle electrodes.
4. Develop staining protocols for a new stain, Luxol Blue, that was obtained.
5. Develop behavioral protocols for ways to measure disease progression and extent.

INTRODUCTION

Background Information

Obesity is one of the world's largest pandemics and health concerns of the 21st century. This disease is characterized by excess body fat and can lead to a number of health concerns, such as heart disease, joint and limb pain, high blood pressure, diabetes, and others. It is clinically defined as a body mass index larger than 30 and biologically, there is more white adipose tissue than metabolically normal.[1]

There are two different kinds of fats, white adipose tissue (WAT) and brown adipose tissue (BAT). These types of adipose tissues have metabolic, cellular, and endocrine functions, and are therefore crucial for energy balance of an organism. WAT acts as an energy buffer in the body, while BAT is highly oxidative and a main contributor to thermogenesis, or the production of heat in the body. An excess of WAT is thought to contribute to obesity due to fat accumulation from energy imbalance, whereas BAT could have protective effects against obesity. There have been studies aiming to "brown" white adipose tissue to combat obesity and prevent diabetes, done by either cold stimulation or pharmacological BAT activation, as adipose is a highly dynamic organ with an exceptional amount of plasticity.[2] However, despite efforts to reverse the effect of obesity, the pandemic continues to grow, and is a worldwide concern, as shown in Figure 1.[3]

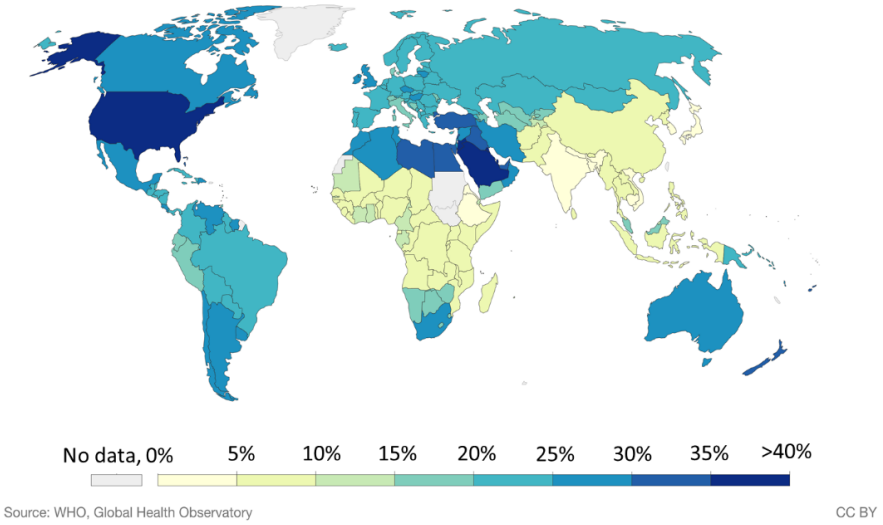

Figure 1. Percentage of adults defined as obese [Body Mass Index (BMI)>30%] as of 2016. Adapted from Ref [3].

Diabetes is one of the world's largest health issues, and is a major disease stemming from obesity. As defined by the Center of Disease Control and Prevention, diabetes is, "the condition in which the body does not properly process food for use as energy".[4] Glucose, or commonly called "sugar", is an essential energy source in the body for many metabolic pathways; however, people with diabetes are unable to properly digest glucose. Insulin is a hormone that enables glucose to get inside the cells of our body for energy, and the pancreas is the primary manufacturer of this hormone. Individuals with diabetes either do not make enough insulin to remove glucose from the blood, or the insulin the pancreas produces does not function at full capacity. As a result, glucose accumulates in the blood and is not taken in by cells for energy. This accumulation can result in serious health effects, including, but not limited to, heart disease, neuropathy, blindness, kidney failure, and even death.[4]

There are two types of diabetes: Type 1 and Type 2. Type 1 diabetes is less common but related to autoimmune, genetic, and environmental factors. Type 2 is much

more common, comprising about 90-95% of diabetes cases, and has multiple risk factors including aging, obesity, family-related diabetes, race, and more.[4] Both types of diabetes are typically caused by the loss of β-cell mass, either physically or functionally. β-cells are crucial for the response of fluctuating insulin demand. They produce insulin and are significantly regulated by glucose to induce metabolic pathways, insulin gene transcription, and insulin secretion. As a result, glucose is essential for normal β-cell function, regulation, and maintenance. However, in diabetes, the functionality and/or quantity of these cells is compromised, resulting in obesity and insulin resistance.[5]

The differences between Type 1 and Type 2 diabetes relates to the mechanism by which glucose is improperly digested and the process in which the β-cells are lost. For instance, Type 1 typically lose β-cells due to an autoimmune process, while Type 2 is the result of insulin resistance.[6] Essentially, in Type 1 diabetes, the patient does not produce enough insulin, so the pathways that allow glucose uptake into the cells is inhibited. In Type 2 diabetes, enough insulin is produced, but the cells are resistant to the hormone because the insulin receptor is insufficient. This is illustrated in Figure 2, which details the differences between Type 1 and Type 2 diabetes and the way that each type affects the body. In general, there is no cure for diabetes, but obesity-related Type 2 treatments can include diet regulation and physical exercise.[4]

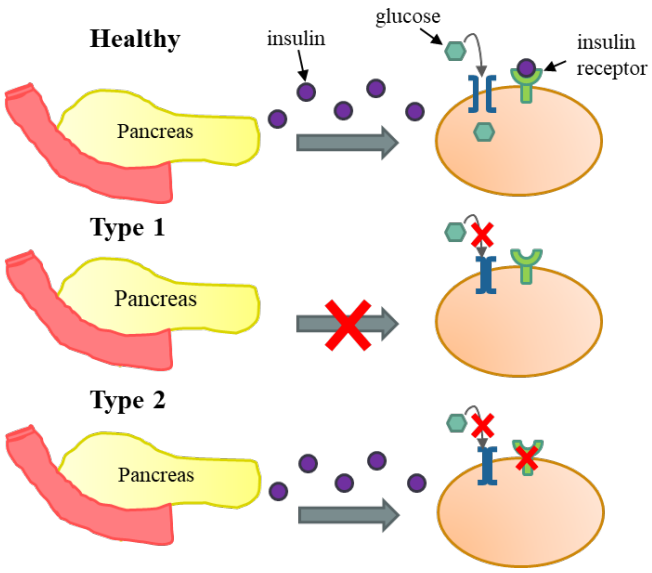

Figure 2. A schematic detailing the differences between a normal, Type 1, and Type 2 diabetic individual and how insulin and insulin receptors play a role in disease.

One of the quintessential symptoms of diabetes is neuropathy at the peripheral limbs, such as an individual's hands and feet. Peripheral neuropathy is the result of damage to the nerves, which can cause things like weakness, numbness and pain; this pain can include stabbing, burning or tingling from the extremities. It is estimated that at least 22 percent of people with diabetes have moderate to severe peripheral neuropathy, and this percentage is only growing.[7] From the 22 percent of individuals with diabetes that will be affected by neuropathy, the current state of care is that there is no cure, just analgesics for pain.[7,8]

Neuropathy can be the result of nerve death associated with nerve axons, myelin sheath, or both, and can involve motor, sensory, or autonomic nerves.[9] In addition to the types of nerves that are involved with peripheral neuropathy, the size of nerve fibers, either small or large, can be used to categorize neuropathy. When the nerves begin to die or become damaged, the communication between neurons is affected, which can lead to

more damage down the road. An individual can either have genetic neuropathy or can acquire this nerve damage over time due to trauma, vascular disorders, systemic diseases, infections, vitamin imbalances, and others. The nerves can become damaged when there is compression of the nerves at the periphery with overuse, repetitive stress, or when there is impeded blood flow to these neurons. A lack of blood flow to the extremities can come from inflammation or blood clots, which ultimately limits oxygen availability and results in nerve damage or death.[10] Other molecular factors that result in nerve death involve toxins or an imbalance of nutrients, such as vitamins, that are critical to healthy nerve function.[10] Again, there are ways to monitor and alleviate symptoms, but there is no treatment to completely reverse the effects of nerve damage and death associated with peripheral neuropathy.

In general, peripheral neuropathy is organized and categorized based on what type of nerve fibers are involved, which includes things like the size and how fast nerves transmit signals along the axon. There are three types of peripheral nerves: somatic motor neurons, somatic sensory neurons, and autonomic neurons. The somatic sensory and autonomic functions are based on whether the nerves are involved in things like touch, pain, temperature, blood pressure, sweating, and heart rate. There are also classifications based on size and diameter of the nerve fibers that are similarly used to classify nerves. A-alpha and A-beta nerve fibers are myelinated and have a large diameter. The function of these nerves is to carry out motor functions and one's sense of touch.[11] Medium-sized diameter nerve fibers that are myelinated are A-gamma nerve fibers which function to transmit the motor function to muscle spindles. Small-diameter nerve fibers that are myelinated and unmyelinated are categorized as A-delta and C nerve fibers, respectively.

These small-diameter fibers carry pain and temperature sensations, as well as transmitting the autonomic functions that are involved in small fiber neuropathy typical with nerve death associated with diabetes.[11]

There are several different types of neuropathies, and diabetic peripheral neuropathy (DPN) is just one broad-encompassing term. One type of DPN is small fiber neuropathy (SFN), which occurs in the small diameter fibers that are both myelinated and unmyelinated. In this type of neuropathy, the large nerve fibers are relatively unaffected by neuropathic pathologies, leaving the small nerves in the hands and feet in pain, but making detection of neuropathy difficult. In SFN, the small diameter fibers such as A-delta and C nerve fibers, which are both myelinated and unmyelinated, are affected directly. This leaves the larger types of peripheral nerve fibers like A-alpha and A-beta to be healthy and unaffected.[11] There are several methods that are used to diagnose SFN, but the techniques are typically insufficient because of the large axons that are targeted, but not affected, in this type of neuropathy.

Addressing small fiber neuropathy in the clinical setting is difficult to interpret and classify. It is defined, loosely, by small fibers with neuropathic pathologies that are more severe compared to the large fiber pathologies. For addressing the severity of SFN qualitatively, a neurologist can look at the extent of dysesthesia.[11] Dysesthesia is a disruption of sensory neurons that can lead to sensations of pain, itchiness, burning, or a lack of sensation in general.[12] Other criterion physicians may use are those that relate specifically to neurological damage in small nerve fibers. This criterion can include a loss of stimuli perception in the extremities like the toes, a loss of the ability to perceive the

environment and the position in that environment (i.e. location and movement), any weaknesses of distal limbs, and the general strength of reflexes.[11]

Similar to DPN and SFN, the adipose cells can also develop neuropathy. Peripheral nerves innervate adipose tissue all throughout the body to communicate with the brain, so adipose neural tissue is also prone to neuropathy. When the nerves die back from the adipose, control of metabolic function is lost, which progresses the obesity with growing adipocyte counts and increased adipose tissue mass. It has been shown and measured that through analysis of both human and mouse tissues, neuropathy does occur at the nerves innervating WAT, so adipose neuropathy is directly related to the growing diabetes epidemic.[13] As a result, knowledge of adipose neuropathy is critical for understanding peripheral neuropathies associated with obesity and diabetes.

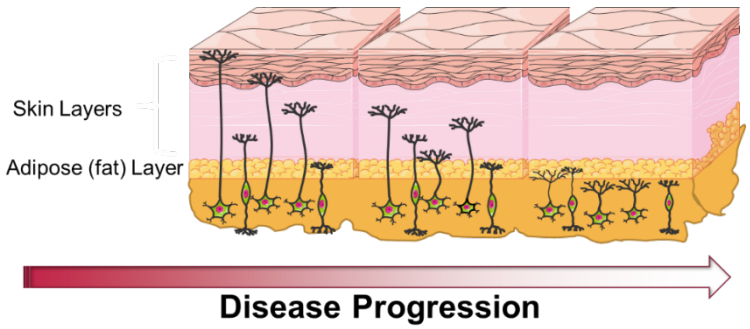

Figure 3. A schematic of peripheral neuropathy nerve death moving from the skin inward as the disease progresses. Reproduced from Magdalena Blaszkiewicz.

Additionally, adipose neuropathy is evidence that nerve death moves from the skin inward, affecting underlying tissues such as subcutaneous white adipose tissue (scWAT). If adipose neuropathy affects adipose metabolic function and control, then nerve death continuing to the underlying tissue could prove to be a mechanism of pain and disease progression.[13] The dying back of nerves in peripheral neuropathy is illustrated in Figure 3, where the sensory skin nerves are affected first and die back to underlying adipose tissue.

Despite having no cure, the prevalence of diabetes is not decreasing; in fact, it is growing. As shown in Figure 4, there are millions of people worldwide affected by diabetes, and the projected numbers of individuals affected with diabetes by 2040 grows in every continent and region across the globe.[14]

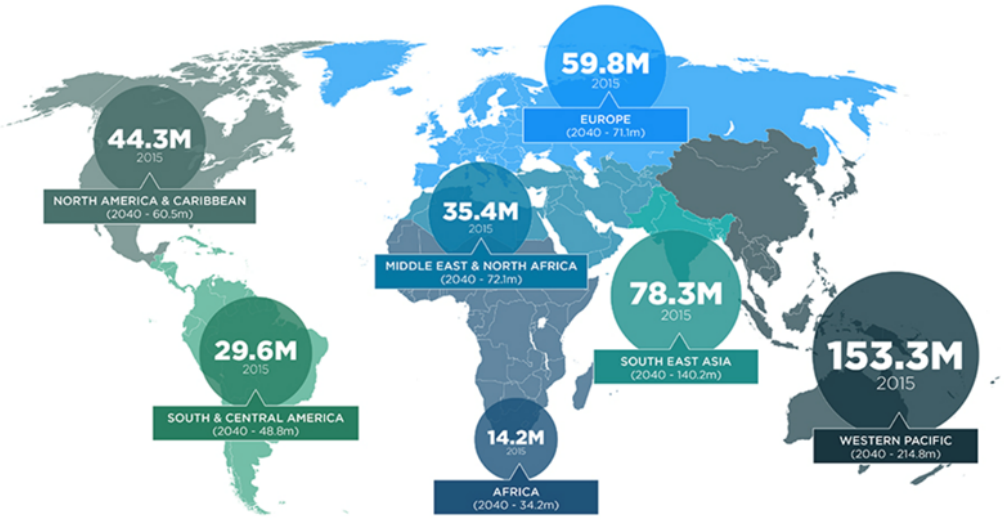

Figure 4. The worldwide prevalence of diabetes across the globe, with the number of individuals affected as of 2015 for each country, and the projected the number of individuals affected in 2040. Reproduced from Ref [14].

With the rising number of diabetic individuals, diagnosis, characterization, and quantification of peripheral neuropathy is in increasing demand because current methods are generally insufficient.

Diagnostic Tests of Neuropathy

The current standard of care for neuropathy diagnosis involves multiple tests, some of which are exceptionally invasive and tedious. The prevalence of neuropathy is growing because individuals are often either unable to diagnose neuropathy, or it is diagnosed at a time where the disease has progressed too far to intervene in an efficient

manner. Additionally, there are currently no cures for neuropathy, nor any way to halt the progression of nerve death. The only way to mitigate pain is with analgesics, and progression may be slowed with and glucose regulation for diabetic individuals.[13] However, there is a clear clinical need for diagnosis of peripheral neuropathy as current methods are inadequate.

One test a neurologist can perform is an electromyography (EMG), which is essentially a test of the connection between one's nerves and the surrounding muscle. This test can be informative to determine if there are problems with nerve signal transmissions or if there is nerve or muscle dysfunctions. An electromyography requires a needle to penetrate the skin to get directly to the nerves, particularly large axons for a larger target, to record how much electric signal is flowing through the muscle.[15] When an individual undergoes this procedure, a small needle or electrode is inserted in the area of interest and an oscilloscope is used to display the electrical activity of the tissue, as shown in Figure 5.[16] Recordings from the patient would be taken while at rest, during a slight contraction, and during a full contraction of the muscle, such as completely extending or bending the knee. The electrical activity recorded is compared to healthy individuals to determine if there is some sort of nerve disfunction, as the neuropathic individual would have a weakened response during nerve stimulation of muscle movement.[15]

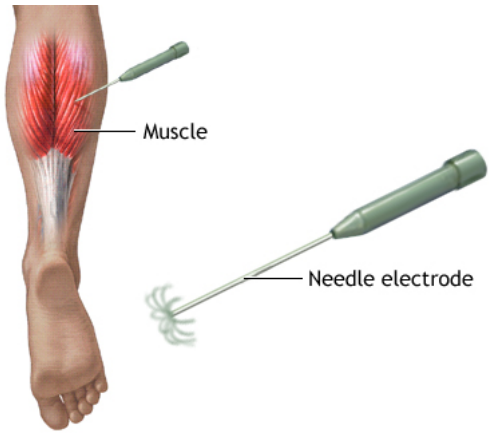

Figure 5. A schematic outlining potential needle and electrode placement for an EMG test. Reproduced from Ref [16].

A test that is typically coupled with an electromyograph to diagnose neuropathy is a nerve conduction velocity (NCV) test. Surface patch electrodes are placed on the skin for stimulation, and others are placed at different known locations to measure the electrical impulse downstream, as shown in Figure 6.[17] The speed of nerve transmission is determined from this test and can be telling of decreased nerve conduction, which can yield information about axonal myelination, or the lack thereof. The electromyograph can help understand the possibility of muscle innervation on these tests, so, collectively, an EMG and NCV can be indicative of general nerve health by comparing the speed of firing or pattern (i.e. latency time) associated with the nerve to healthy individuals to see if these factors are affected by neuropathy-associated nerve damage.[15]

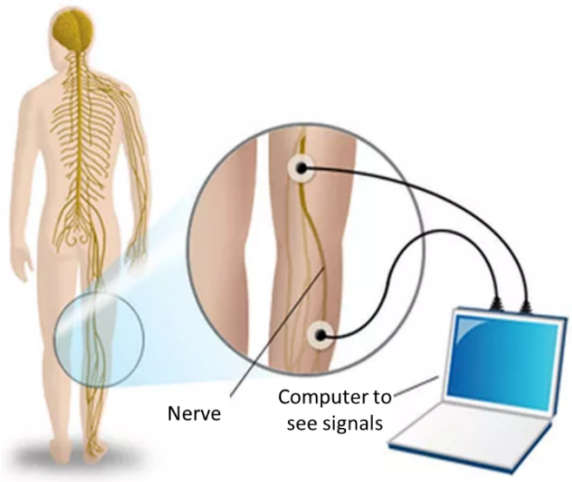

Figure 6. Nerve conduction velocity procedure setup to show potential surface patch electrode placement. Adapted from Ref [17].

An EMG and NCV test can be useful for extensive nerve damage, but both are invasive and not a guaranteed diagnosis of small fiber neuropathy (SFN). The area of needle penetration for an EMG can be sore for several days afterward, similar to the feeling of receiving a shot. Multiple needles may be necessary for diagnosis, so the discomfort is more than just a single needle in one spot. Bleeding and bruising can also occur, and the use of needles poses the threat of infection.[15] In addition, receiving these tests is inconvenient, as the patient must see a neurologist or other specially trained individual; these specialized people are often booked many months in advance. Additionally, the results of these tests vary with age; younger individuals generally have less myelination than a healthy adult, so the nerve conduction speed in younger people is typically slower. Therefore, bridging the gap between age and actual neuropathy-like symptoms is a potential limitation of these tests. The electrodes used for an EMG and NCV are sensitive to noise and temperature, among other factors, which could influence the diagnosis. If the temperature of one's skin is colder, then the nerve conduction speed

and muscular electrical activity can be hindered and affected in a way that could indicate neuropathy, but in a healthy individual.[18]

Nerve conduction velocity tests are the typical diagnostic tools, but NeuroMetrix has developed a less invasive device to evaluate diabetic peripheral neuropathies called the DPNCheck. The NCV of the sural nerve, the main nerve through the calf, and the response to stimulation is measured and analyzed for neuropathy diagnosis. This device, shown in Figure 7, is fairly sensitive to changes in the functionality of the sural nerve.[19] However, by the time the neuropathy spreads from the feet to the calf nerves that are measured with this device, it is too late to treat. Additionally, the progression of neuropathy to the calves is significantly more painful for the diabetic individual, and such pain will only continue to worsen without earlier treatment.

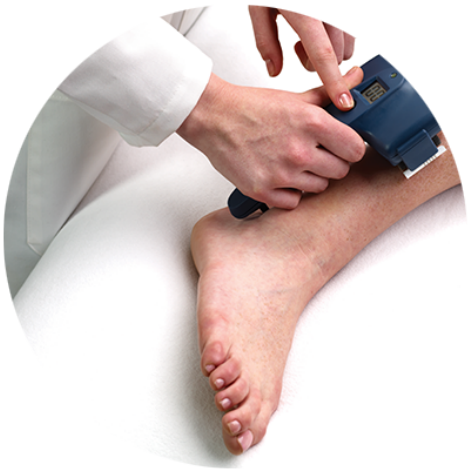

Figure 7. DPNCheck by NeuroMetrix is a device targeted at measuring sural nerve conductance. Reproduced from Ref [19].

Another tool that is currently used to diagnose neuropathy is a skin biopsy. Skin biopsies are typically used for small nerve fiber neuropathy, which is one of the most difficult to distinguish and recognize. When a patient is receiving a skin biopsy, the area is often numbed with lidocaine and the doctor removes a sample of skin. These samples

can be taken from any part of the body, such as the fingers and toes, unlike an EMG or NCV. The collected skin sample is then analyzed with bright-field immunohistochemistry or indirect immunofluorescence to analyze the small nerve fibers from that tissue.[20] When sectioned and stained with PGP 9.5, a pan-neuronal marker, the analyst would be able to observe the nerves present and do necessary calculations on the tissue to diagnose peripheral neuropathy, such as density counts per area.[20]

Although the pathologist that receives the stained sample could visualize the nerves, this technique is not ideal for diagnosing small nerve fiber neuropathy. Skin biopsies are very invasive, as they require a skin sample to be scraped and removed from the patient.[20] Additionally, this technique only assesses morphologies of the nerves instead of function. Observation of the nerves in the biopsy that are stained can give subjective results if there are discrepancies in opinions of the viewers based on the small sample size taken. To really diagnose and assess the extent of neuropathy, ideally, one would be able to address function *and* morphology.

Yet another method that is used for neuropathy diagnosis is microneurography. Microneurography is used to directly record and measure sympathetic action potentials. This technique employs skin-surface electrodes, so it is minimally invasive, and can measure peripheral nerve conductance and assess the function of small nerve fibers. The spectrum of signals collected is relatively broad, so multiple nerve types are distinguished. However, microneurography fails to record sympathetic activity for diabetic individuals due to potential sensitivity limits. Therefore, this could significantly limit the types of patients that can benefit from this diagnostic tool.[21,22]

A relatively new approach of diagnosing peripheral neuropathy is to address sudomotor function, or how well an individual's sweat glands are functioning. The thin, unmyelinated C fibers innervate sweat glands, and these are the types of fibers that are more greatly influenced in diabetic neuropathy and SFN. C fibers are affected early in diabetes and can continue to worsen as the disease and neuropathy progresses. Therefore, measuring sweat gland dysfunction could be useful to determine the function and integrity of the innervating and surrounding C fibers.[23] One device, named Sudoscan, has been engineered to diagnose sweat gland dysfunction. This method is minimally invasive and pain free, gives a functional assessment of the small fibers, and can detect early neuropathy before symptoms arise.[23]

The Sudoscan device consists of two electrodes that one's hands or feet are placed on. When an individual has healthy sweat gland dysfunction, and the small fibers are innervating these glands, chloride ions are secreted and released to the surface.[24] The electrodes for the Sudoscan device measures chloride ion conductance, so a high conductivity would indicate more sympathetic innervation; therefore, the progression of prediabetes and neuropathy can be determined based on chloride conductivity.[24,25] Although this is an indirect functional assessment of the small nerve fibers, peripheral nerve conductance cannot be measured, and Sudoscan does not provide an analysis of many nerve types that can be affected with peripheral neuropathy.

Despite several different types of diagnostic techniques and treatments, these tend to be insufficient in accurately addressing peripheral neuropathy. Methods such as skin biopsies, nerve conduction velocity tests, and an electromyograph are invasive and can cause pain for the individual. Additionally, tests like NCV, EMG, and the DPNCheck

device can measure nerve conduction velocities of large axons; however, by the time the neuropathy reaches these points, it is often too late for intervention and alleviation of the pain. Other methods, such as Sudoscan, are indirect measures of neuropathy and could be insensitive to small changes as the neuropathy progresses from the skin inward. Therefore, there is a clear need for a noninvasive and effective measure of peripheral neuropathy detection at the early stages of disease that emphasizes on addressing nerve functionality. A promising method for this diagnostic platform is microneedles.

The Growing Use of Microneedles

Microneedles are an emerging technology in biomedical engineering. Microneedles are arrays of small, minimally invasive needles that have various uses in many industries, such as for transdermal drug delivery (TDD). Microneedles can be hollow to administer drugs to bypass the tough epidermal layer, making the speed of drug delivery directly into the subcutaneous tissues greatly enhanced, but also the amount that is administered to the individual. A general schematic of TDD is illustrated in Figure 8, where small drugs can be released directly into the body through the hollow microneedles due to increased skin permeability and more direct drug administration.[26]

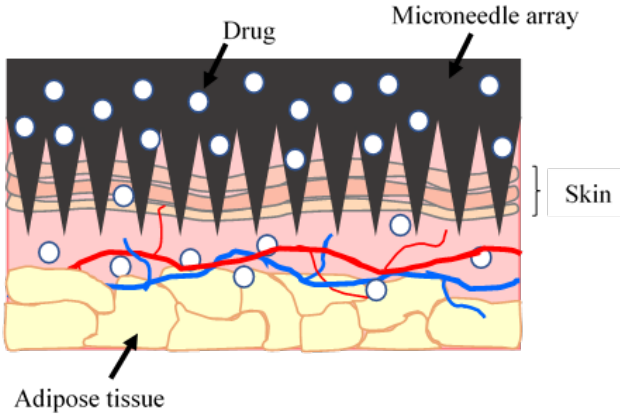

Figure 8. A schematic illustrating transdermal drug delivery with a microneedle array.

Microneedle arrays are preferred over single needles because they are painless and minimally invasive. There is increased microneedle use in research because there are a variety of materials and structures that they can be made of, allowing for very diverse uses. There are microneedles made of a variety of materials including, but not limited to, silicon, glass, ceramic, hydrogel, polymer, and metal. The length of the needle can also be changed by etching and sawing the needles in a particular fashion.[27] Additionally, the needles can be coated with insulation or other materials to further optimize their use in research. Therefore, there is a growing application of microneedles because they are relatively customizable due to the characteristics that can be modified, and hold promise for many different applications.

In the creation of a device for early diagnosis of peripheral neuropathy, microneedles seem promising. Using microneedles to record electrical signals is a relatively novel approach as many researchers and industries are not conducting these kinds of experiments. However, coating or making microneedles in a conductive material enables them to be used as electrodes, which have the ability to measure electrical signals. Biomedical companies such as BlackRock Microsystems sell arrays to measure nerve conductance, but typically for brain recordings.[28,29] Many experiments that involve microneedles recording electrical signals in the body are done with nerves in the central nervous system like the brain and spinal cord, rather than the peripheral nervous system. Blackrock Microsystems also creates a slant array for peripheral nerve data, but similar to the diagnostic tests for neuropathy, these arrays are targeted for implantation in nerves like the sciatic and sural nerves in the leg, rather than small fiber nerves at the extremities that are affected by peripheral neuropathy first.[30] Therefore, the design of this novel

neuropathy diagnosis device is to use microneedles to record electrical signals from the *extremities* to determine the functionality of nerves. To do so, an understanding of neurophysiology and what can be affected by neuropathy is necessary.

Neurophysiology Overview

Neurophysiology is a branch of neuroscience and physiology that focuses on the physiology and functioning of the nervous system; this field of study often involves electrophysiological or other biological tools. Neurons function by establishing electrochemical gradients across the cell membrane to generate action potentials; essentially, neurons make use of an established transmembrane voltage difference. In current nerve conduction studies, typically the voltage of an action potential or the speed of transmission is measured. It is known that the resting membrane potential of a neuron is about -40 to -90 mV. When a neuron is stimulated or information via synaptic terminals is sent, the membrane potential can depolarize, resulting in an action potential.[31] Measuring the amplitude of voltage from the action potential is one way in which researchers test the functionality of nerves because it can be done locally. Additionally, an action potential travels down the axon at a particular speed for that neuron, depending on the extent of myelination of that nerve. Determining any changes in action potential propagation velocity is another way one can assess nerve function, especially across a larger area. Therefore, voltage amplitude and velocity are two characteristics that are employed in neurophysiology because these characteristics give information both locally and across a larger portion of nerve.[31]

It is known that neuropathy can decrease the amount of myelination around a nerve.[9] The myelin sheath is an insulating layer around the nerve that speeds up nerve transmission. In a neuropathic individual, the amount of nerve myelination is less, which would ultimately slow down the nerve conduction velocity. In a study of 500 diabetic individuals, the motor and sensory nerve conduction in patients with diabetic peripheral neuropathy was measured and compared to those of healthy people. It was concluded that distal motor latency (DML) in the median and common peroneal nerves in symptomatic diabetics was longer than that of asymptomatic diabetics, potentially indicating less myelination. The compound motor actional potential (CMAP) amplitude in these same nerves was also lower in the diabetic individuals suffering from peripheral neuropathy compared to diabetic individuals with no neuropathic symptoms, as shown in Figure 9.[32]

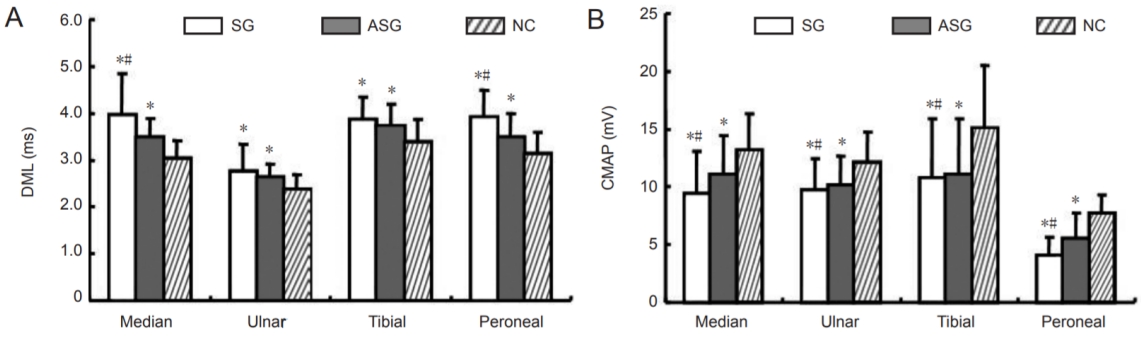

Figure 9. Motor nerve conduction in diabetic patients with and without peripheral neuropathy and in control subjects. Figure 9A is the Distal motor latency (DML) and Figure 9B is the compound muscle action potential (CMAP) between symptomatic groups (SG), asymptomatic groups (ASG), and control groups (NC). Reproduced from Ref [32].

Sensory nerve conduction studies were also done in this study of 500 diabetic individuals. It was determined that sensory nerve action potential amplitude (SNAP) and sensory nerve conduction velocity (SCV) in the diabetic groups (symptomatic and asymptomatic) was lower than that in healthy controls. Furthermore, in the symptomatic

diabetic group, the SNAP and SCV of the median, ulnar, posterior tibial, and common peroneal nerves were significantly lower in the symptomatic group compared to the asymptomatic group, as detailed in Figure 10.[32]

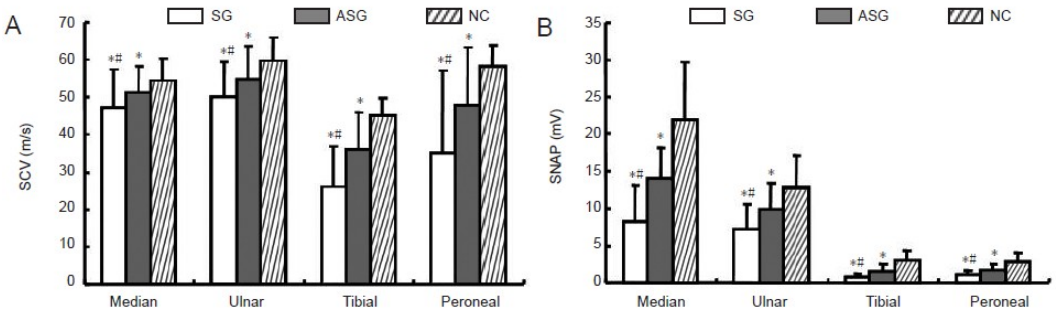

Figure 10. Sensory nerve conduction in diabetic patients with and without peripheral neuropathy and in control subjects. Figure 10A illustrates the sensory nerve conduction velocity and Figure 10B illustrates the sensory nerve action potential between symptomatic groups, asymptomatic groups, and control groups. Reproduced from Ref [32].

Therefore, while conducting neurophysiology tests with the designed microneedle array, the latency, action potential amplitude, and conduction velocity are all aspects that should be considered when configuring electronics and analyzing data. These three characteristics are factors that will be affected by peripheral neuropathy; as a result, differences in electrical signals of peripheral nerves between diabetic and control groups can be compared to obtain an early diagnosis and track the progression of disease. The expected neurophysiological results of the latency, conduction velocity, and the action potential amplitude of a diseased versus healthy individual is illustrated in Table 1.

Table 1. Summary of expected neurophysiological changes between a healthy and diabetic individual, relative to each other.

Characteristic	Control	Diabetic/Neuropathic
Latency	↓	↑
Conduction velocity	↑	↓
Action potential amplitude	↑	↓

Using Mice as a Model of Disease

There are several organisms that are used in research and industry to conduct preclinical tests or to model human mechanisms, including mice, zebrafish, rats, monkeys, fruit flies, and more. Each organism has its own benefits depending on the area of study; for instance, zebrafish are useful in studying the immune system due to similar human cell types, such as neutrophils. However, mice are one of the most common research animals, and there are several reasons why. Most importantly, mice and humans have many of the same genes, and humans and mice develop in the same way from an egg and sperm. As a result, the organ systems of mice are closely related to that of humans, and this similarity allows researchers to study biological mechanisms and the effects on the entire body. Another reason the mouse is used is because of their lifespan. Mice live long enough to be able to easily conduct longitudinal studies of chronic diseases or aging, but short enough to be reproducible. The small size of mice makes them easy to house and maintain for the entirety of their life.[33] Also, much is known about mice and their genome, so it can be easily manipulated to observe changes, and different strains can be more effective than others, depending on the study's purpose. Therefore, in the early stages of prototyping and designing the device, mice will be used as a model of disease.[33]

In the Townsend Laboratory, mice are used as a model of disease as they develop neuropathy either genetically or through administration of a high fat diet. The genetically induced neuropathy model is a BTBR *ob/ob* leptin-deficient diabetic mouse. The BTBR model develops severe Type 2 diabetes because the animals are genetically insulin resistant. This insulin resistance and other factors associated with diabetes are developed,

making the BTBR *ob/ob* mice a model for disease progression as the diabetes advances over the lifetime of the mouse.[34] Additionally, the BTBR *ob/ob* model can be used to test the first prototype of this diagnostic device as these genetically altered mice do develop peripheral and adipose neuropathy.[13]

Mice develop diabetes not only genetically, but also environmentally through the administration of a high-fat diet. In a study where mice were fed a high-fat diet for 12 months, the researchers found diabetic morphologies related to insulin levels, glucose tolerance, and more. Therefore, mice can develop diabetes, and consequently peripheral neuropathy, while being fed a high-fat diet similar to humans and the Type 2 diabetes epidemic.[35] As a result, the designed early-diagnosis prototype is using mice for initial tests and optimization, and eventually as a method to track disease progression.

Initially, much work has gone into creating and optimizing a device to use for a longitudinal study in the future. The electronic components used must be set up appropriately to reduce noise but maximize reproducibility. Protocols, standards, and various tests are described and conducted to learn about the needles, software, and working with neurophysiology in a practical sense to develop a point-of-care diagnostic device to translate to human clinical trials.

PROTOCOL DEVELOPMENT

With the aforementioned project goals, most of the work done for this thesis was developing an appropriate protocol to use the new equipment. BIOPAC, the recording hardware and software, was new to the Townsend Laboratory; therefore, it was essential to get familiar with it and develop a working protocol to take recordings. To do so, much trial and error was done with the electrodes using saline agar solution and horseshoe crab tissue. The amplifier settings were altered to see how the resulting current was affected in many different experiments to further understand the recording signals and how to manipulate the hardware. Additionally, much work was put in to understand how to use the AcqKnowledge software that is used to look at and analyze the signals. All recordings and experiments conducted in this thesis used the developed protocol after much troubleshooting and manipulation had been done.

Furthermore, behavioral protocols to test for disease progression and extent were developed and tested. A cold plate test and an acetone test will eventually be used in a longitudinal study in mice to behaviorally measure neuropathy, and these tests were new to the Townsend Laboratory. After Institutional Animal Care and Use Committee (IACUC) approval, these protocols we engineering to eventually be used as a neuropathy test.

Another protocol that was developed as part of this thesis work was to use Luxol Fast Blue. This is a stain for myelinated nerves, and again was new to the Townsend Laboratory. In order to optimize a staining procedure, many tissues, such as muscle, adipose, and sciatic nerve, were stained. All these tissues were stained multiple times

with multiple different incubation times in order to optimize the protocol to be used. As a result, a large part of this thesis work accomplished its goals for protocol development that will be used for experiments and results elaborated on below, but also in the future for comparison methods of neuropathy.

MATERIALS AND METHODS

Materials and Hardware

- BIOPAC MP160 Data Acquisition Hardware, Part No. MP160WSW
- BIOPAC AcqKnowledge® 5 Acquisition and Analysis Software
- BIOPAC HLT100C Transducer module
- BIOPAC AC150A Power Supply
- BIOPAC MCE100C Micro Electrode Amplifier (x4)
- Alligator clips
- Laptop
- VisionTek USB Docking Station
- Power strip and power supply
- Mini hook leads
- Faraday cage
- Dissection microscope with light
- Custom Microneedles*
- Microprobes 0.5 MΩ commercial platinum iridium electrode
- Natus Ultra Disposable Stainless-Steel Subdermal Needle Electrodes

**Due to current intellectual property and the patent process, certain aspects and dimensions of the needles, as well as some specifics of the work conducted must be omitted*

Methods

I. Animal Subjects

Animals used in this work include *Limulus polyphemus*, or horseshoe crab, under the assistance of Dr. Leonard Kass. *Limulus* tissue was used to validate the recording setup and electronics by using a well-established and well-known nerve model. Mice were used with IACUC approval and regulations, detailed in Appendix A. During basal recordings tests (no stimulation), mice were anesthetized with 1.5% isoflurane in pure O_2 at a flow rate of 1 L/min and euthanized after nerve recordings by trained personnel.

II. Experimental Setup

All recordings were taken in the Faraday cage illustrated in Figure 11. The BIOPAC hardware, laptop, and USB docking station are all positioned on a table next to the Faraday cage to minimize the length of wires between the recording area and the hardware, therefore reducing noise in the recordings. Electrodes that are being used for

Figure 11. Recording setup with the Faraday cage, BIOPAC hardware, and the laptop with the necessary software. Reproduced from Brooke Villinski.

recording are connected to red mini-hook leads, detailed in Figure 12, and there are marked ground and stimulation electrodes that come from the respective black leads.

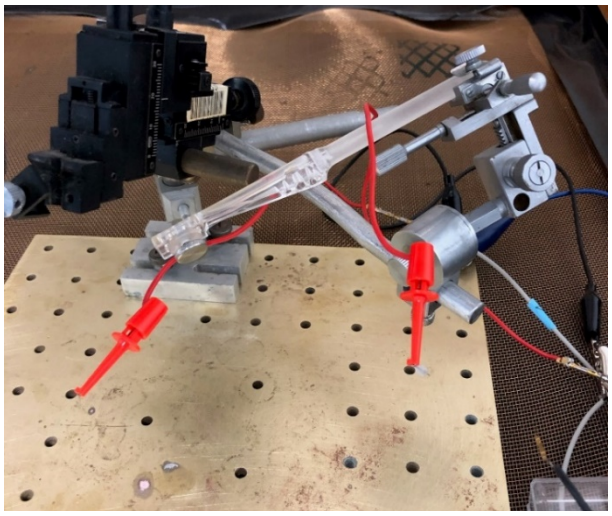

Figure 12. Red mini-hook leads that hold all differential recording electrodes.

III. BIOPAC Protocol for Collecting Recordings

There are many functions of the BIOPAC software and hardware, and it can execute multiple things both in the collection and analysis of data. There are recordings with and without stimulation, both of which are used to collect the data presented in this thesis.

First, to use the software, turn on the VisionTek external USB connector, the laptop, and the BIOPAC MP160 hardware. Insert the teal AcqKnowledge USB into the VisionTek connector to have the hardware communicate with the software and laptop. When the computer is turned on, open the AcqKnowledge icon on the desktop to open the recording software. Create a new experiment, and then open an existing graph template file (GTL). The files are named according to their settings, such as the stimulation voltage amplitude, duration, or for simple basal recordings. Opening this GTL will load the saved settings within the file. Before recording, ensure amplifier

settings are appropriate on the MCE100C hardware. The standard settings for recording with and without stimulation are detailed in Table 2, unless specified otherwise.

Table 2. Standard MCE100C amplifier settings.

Setting	Value on MCE100C Amplifier
Gain	1000x
Low Pass (LP) Filter	3kHz
High Pass (HP) Filter #1	100kHz
High Pass (HP) Filter #2	0.5 Hz HP

Inside the Faraday cage, the alligator hook leads are set up for recordings by placing electrodes in the desired tissue. The recordings are from a differential amplifier, so two electrodes are put into the tissue, with a ground to reduce noise. A stimulation electrode can also be put into the tissue close to the recording electrodes, but care should be taken to ensure all leads are appropriate for their purpose (i.e. differential recording and stimulation electrodes lead to the amplifier at the correct position). After electrodes are inserted, the Faraday cage can be closed, and “Start” can be selected on the AcqKnowledge software. This setup was used to record for all experiments involving recordings, with and without stimulation. Different electrodes were incorporated to compare among needle types, but the general configuration of the electrophysiology rig is the same. A detailed procedure is outlined in Appendix B.

IV. Cold and Hot Plate and Acetone Testing

Part of the platform is to ultimately record electrical signals from the mouse and relate the results to already-established measures of neuropathy. Currently, hot and cold surfaces are used to test for the presence of neuropathy. If a subject is diabetic, the response time will either be slower or completely diminished when exposed to hot or cold temperatures compared to a healthy individual. Additionally, chemical tests, such as acetone, are used for similar qualitative responses to the acetone being applied to the hind paw. In a future longitudinal study with the designed needle array, all three tests will be used to relate electrical signals to the progression of neuropathy. These tests were new to the Townsend Laboratory, so protocols had to be revised and established to conduct these tests. Therefore, part of the project included the development of these protocols. The established protocols for the cold plate and acetone tests are shown in detail Appendix C. Also, in addition to the protocols, a template for recording data for the cold plate test was established. This template is also detailed in Appendix C.

RESULTS

I. Using *Limulus* Tissue to Validate the Electrical Setup

Limulus tissue is a well-established nerve model, so the initial experiment was to validate the electrical setup and the wire configurations. Additionally, BIOPAC was a new software, so a protocol needed to be established on how to use the hardware. Dead *Limulus* tissue was obtained and the custom needles to be used in the future array were used for stimulation of the tissue. Standard commercial Microprobes electrodes were used for differential recordings, and each needle electrode was positioned as detailed in Figure 13. In the experiment, electrodes were moved from one position, termed Setup 1, to further apart in Setup 2 in Figure 13 to measure differences in recordings amplitudes.

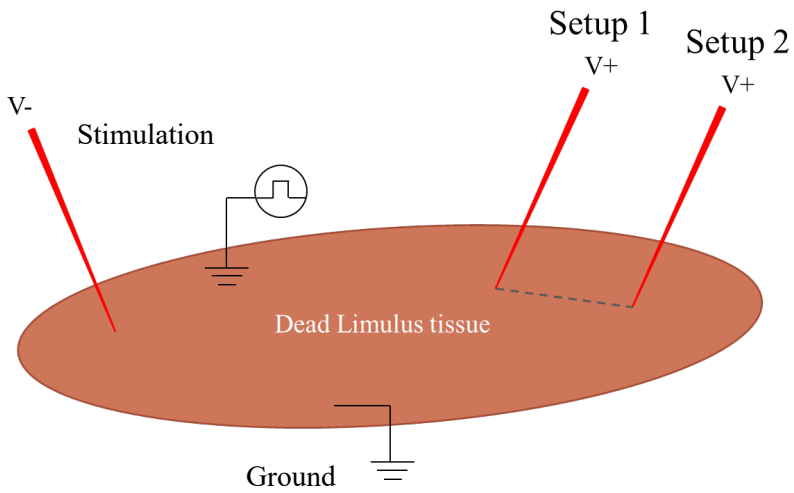

Figure 13. Electrode setup in dead Limulus tissue to validate electrical configurations and observe differences when electrodes are separated by two different distances.

Sample recordings are illustrated in Figure 14. Setup 1, where the recording electrodes were closer together, has a higher signal amplitude compared to Setup 2, where the recording electrodes were further apart. This is what was expected as when the needles are closer together, less signal is lost to the surroundings. This experiment was a

proof-of-concept test to ensure recording electrodes, wires, and the BIOPAC hardware was set up appropriately, and to become familiar with how to use BIOPAC. A protocol, detailed in Appendix B, was established as part of this test. Therefore, it can be concluded from this experiment that moving forward, the software and hardware setup is set up appropriately to measure recordings in tissues.

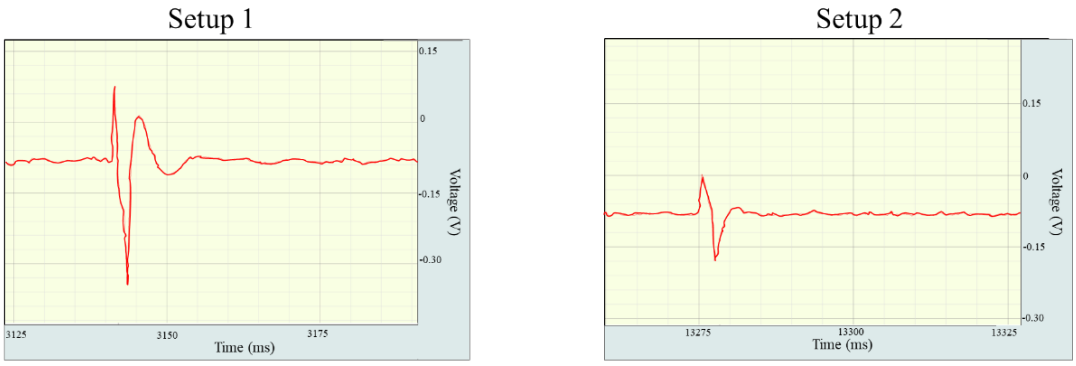

Figure 14. Sample recordings from dead Limulus tissue with electrodes placed in different positions in Setup 1 and Setup 2. Setup 1 has a higher voltage amplitude compared to Setup 2, which is expected given that Setup 1 needles are closer together.

II. Using *Limulus* Tissue to Learn How to Stimulate with the Software

Basal and stimulated recordings are desired for the final prototype of the device, so learning how to stimulate with BIOPAC was essential. The purpose of this experiment was to further develop a protocol and become familiar with stimulation using the software. Again, *Limulus* tissue was used because of its reputation as a well-established nerve model. Recording electrodes were placed into two ends of the tissue, with a ground and a stimulation electrode in between, detailed in Figure 15. The purpose of this experiment was to test different stimulation voltage magnitudes to measure any limits in voltage signal, to, in the future, stimulate with the lowest possible voltage to minimize

risk to the animal. Four stimulation voltage amplitudes were tested: 0.05 V, 0.1 V, 0.2 V, and 0.4 V.

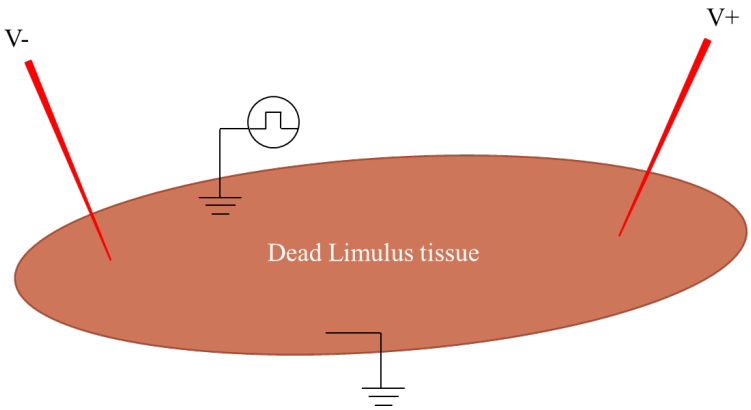

Figure 15. Electrical setup used in the experiment to measure different stimulation voltage levels.

Sample recordings from the experiment with different stimulation voltages are detailed in Figure 16. As the stimulation voltage increased, the amplitude of the signal also increased, as shown in Figure 16A, 16B, and 16C. From Figure 16C and 16D, there is a potential saturation in the signal, as there is no observable difference between 0.2V and 0.4V stimulation. However, the results are still consistent with what one would expect with an increase in the stimulation voltage; therefore, a method to stimulate with the BIOPAC hardware and software was established and incorporated into the protocol in Appendix B to, in the future, stimulate animal subjects.

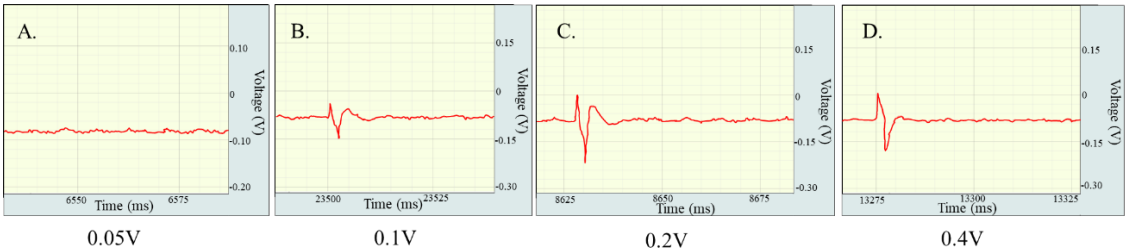

Figure 16. Sample recordings from the experiment with stimulation voltages of A) 0.05V, B) 0.1V, C) 0.2V and D) 0.4V. As the magnitude of stimulation increased, so did the signal, until a plateau at 0.2V and 0.4V.

III. Testing the First 3D Printed Microneedle Array

(array designed and printed by Julia Towne)

The first prototype of a device was designed and printed, so the purpose of the experiment was to test this design and its dimensions on a euthanized mouse. A mouse was euthanized and basal recordings were taken from the shaved scWAT area, with the electrodes positioned in the image in Figure 17. The dimensions of the needle array were 3.5cm x 1.5cm with needles spaced by 5.1 mm apart, with a penetration depth of 1 mm.

Figure 17. Image of the mouse and needle array positioning that was used in the experiment to test design dimensions and recording from a needle array. Array designed and printed by Julia Towne.

In this experiment, basal recordings were taken by moving one differential recording electrode around all nine different needle positions in the array. The needle positions were labeled according to Figure 18, and all combinations of needles in relation to Electrode 1 were tested to determine if noise levels were appropriate to distances between needles; again, this was to show that the BIOPAC software and hardware was positioned appropriately and to demonstrate how recordings from an array could be done.

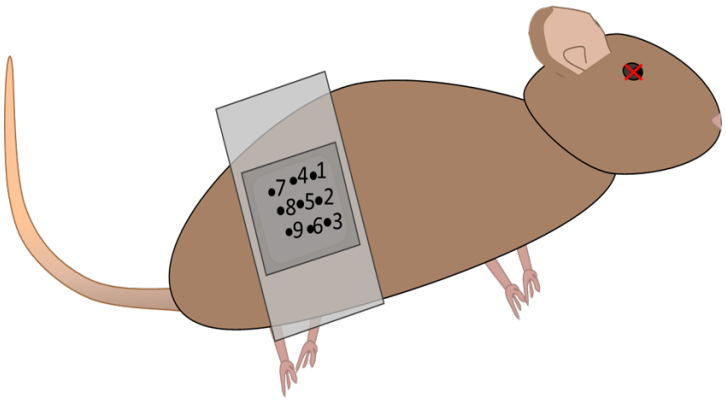

Figure 18. Electrode labels on the mouse that were used in the experiment to test different recording positions.

Sample recordings from this experiment are shown in Figure 19. When the electrodes were moved to the same needle, Needle 1, the signal canceled out as expected in Figure 19A. When the electrode was moved to positions 1 and 2, essentially right next to each other, there was an increase in signal, as shown in Figure 19B. The recording electrodes were then moved as far apart as possible within the array, to positions 1 and 9. As expected, when the needles were moved further away, the noise increased, as detailed in Figure 19C. The noise was expected to increase because the recordings were just basal recordings, so the amount of tissue between the electrodes increased, thus increasing the amount of noise. Therefore, from this experiment, it could be concluded that a needle array is feasible to obtain recordings from different positions within the array.

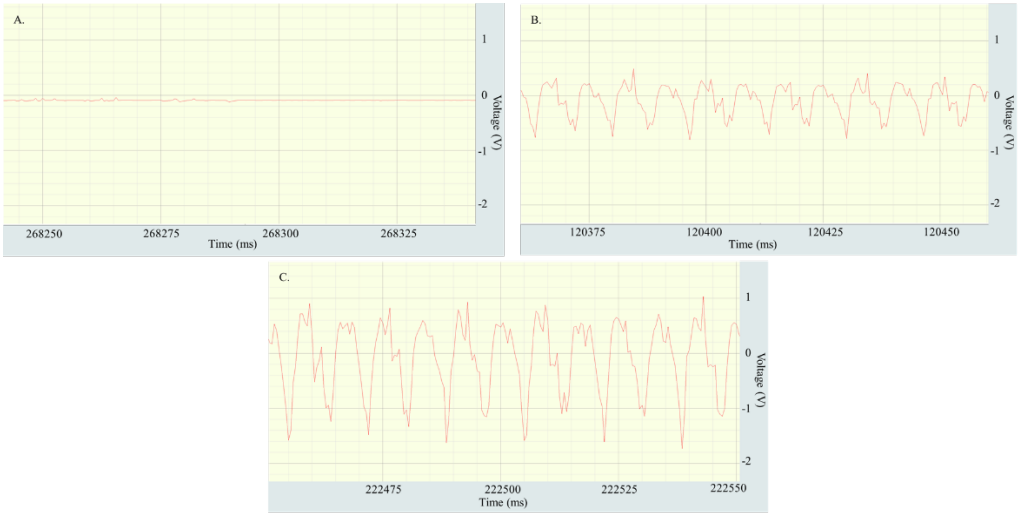

Figure 19. Sample recordings from the first printed device. Electrodes were moved to A) Positions 1 and 1, B) 1 and 2, and C) 1 and 9. As the distance between needles increased, the amount of noise also increased, as expected.

However, another purpose of the experiment was to test the overall design and dimensions of the device. The dimensions of the array that were printed were concluded to be too large moving forward, because the needles often moved out of the skin. The spacing between needles, 5.1mm, made the array to cover an exceptional amount of the entire mouse, even though the scWAT was the target area. The large rectangular design on the rounded mouse made needles leave the surface of the skin, causing issues with the needle staying positioned on the mouse and needles continuously penetrating the skin. Another aspect of the array that could better facilitate needle penetration is increasing the depth of needle exposed. In this design, 1mm of needle was exposed to pierce the skin. In future prototypes, this could be increased to 2mm to go deeper into tissue and to help with maintaining needle penetration for the entire experiment. Additionally, in Figure 17, the design featured a large cylindrical rod from the center of the electrode. This rod was incorporated into the design with the idea it would help apply even pressure to the array when the needles were being inserted into the mouse. However, the rod was unnecessary,

and even obstructed the movement of some electrodes around the array. As a result, from the initial tests of the device, can be concluded that the dimensions of 3.5cm x 1.5cm with needles spaced by 5.1 mm apart and a penetration depth of 1 mm is not suitable for future designs. The array should be shortened with needles positioned closer together, and with the rod from the center removed. This experiment was important and informational for determining and testing design dimensions to move forward with designed and printed prototypes.

IV. Basal Mouse Recordings to Compare Needles to Commercial Needles

The first prototype of the design uses needles that are not currently used for recording electrical signals in tissue. As a result, it is essential to learn about these needles and to compare these needles to commercially available electrodes that are used for neural recordings. The purpose of this experiment was to use an anesthetized mouse to obtain basal recordings of the subcutaneous adipose tissue using both the design needles and commercially available needles to compare signals between the two. Amplifier settings were also modified with the needles in the tissue during the experiment to further understand how high and low pass filters affect signal.

First, one recording custom needle was inserted into the scWAT of the mouse, as shown in Figure 20. The other differential recording electrode, not shown in the figure, was inserted in the base of the tail. Recordings were taken with the standard amplifier

settings but were changed throughout the procedure to try and reduce noise of the produced signals.

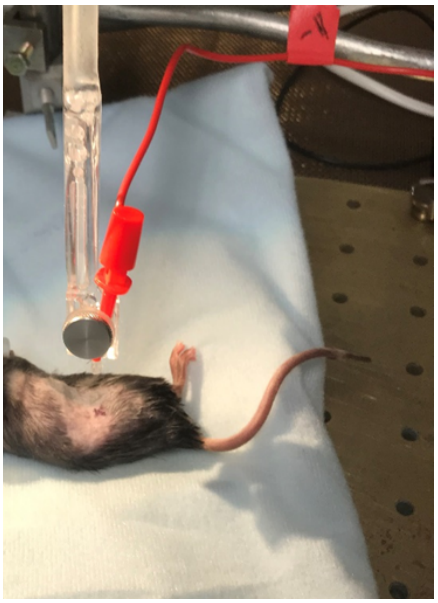

Figure 20. Custom needle recording setup used in the experiment to get basal signals from an anesthetized mouse.

After recordings were taken from the custom needles, the recording electrode was switched to a 0.5 MΩ commercial platinum iridium electrode from Microprobes, where the electrode details are shown in Figure 21.

Figure 21. Microprobes recordings electrode that was used to compare to custom needles in an anesthetized mouse.

The electrical setup was the same for the Microprobes recordings as the custom design needles. One recording electrode was placed at the same location in the scWAT, and the other recording electrode placed in the tail, as detailed in Figure 22. The Microprobes for Life Science is a company that focuses solely on neural microelectrodes, needle arrays, and more. The needles from Microprobes are distributed worldwide for neural recordings, so these electrodes were selected as a comparison to check the quality and reliability of signals from the custom needles.

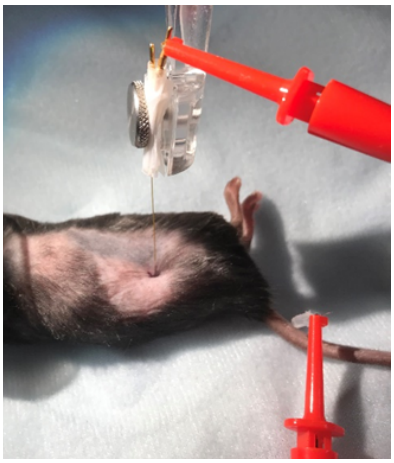

Figure 22. Microprobes setup showing the two differential recording electrodes, with one in the tail that stayed constant throughout the experiment. The other needle was switched with the custom needles for comparison.

The Microprobes needles used in this experiment are not beveled like the custom needles are, so inserting them into the mouse tissue was much more difficult. Mouse skin is very flexible, so upon insertion of the needle, the skin had to be pulled quite taut for the needle to even slightly penetrate the skin. The custom electrodes are three-beveled needles, which makes insertion into the tissue much easier. As a result, comparing electrical recordings from these two needles is difficult, given the Microprobes needles may not have even penetrated the tissue. This is a consideration that is important to consider for future iterations of the designed prototype, given that beveled needles are

much easier to work with compared to already available needles. The design, then, should incorporate beveled needles when being put into an array to record electrical signals faster and with more ease.

V. Natus and Custom Needle Basal Recordings in Anesthetized Mouse

An important aspect of the design platform is to make sure the custom needles that will be used will be comparable to other needles, and yield results like what one would expect. The objective of this experiment was to compare the feasibility of collecting basal recordings from a set of commercially available needles to the custom needles in an anesthetized mouse. Natus needles are currently used in nerve conduction velocity tests, so they are a well-established comparison. This comparison between the two needle types was done in three different tissues to determine if there are any differences between the two needles, or any differences between tissue types. The tissue types tested for each needle was the scWAT, muscle tissue, and the paw. Subcutaneous white adipose tissue and the paw are two areas that will be tested in the future with the first fully developed prototype because these two areas are prone to peripheral neuropathy. A sample of the electrical setup in the scWAT tissue is illustrated in Figure 23, where the custom needles are inserted into the tissue.

Figure 23.Sample image from the experiment tested different tissue types with custom needles inserted in the scWAT.

Electrical signals from the muscle were tested in the experiment to compare neuromuscular junctions between needles. The neuromuscular junction is a very well-studied and well-understood electrical signal, so relating signals between the electrodes would be suitable. Another purpose of this experiment was to further reduce noise levels inside the faraday cage, as this is one of the most critical aspects when recording from nerves. The neuromuscular junction, then, would ideally give insight on how much noise must be further tested and reduced.

From this experiment, it can be concluded that both the Natus needles and the custom needles are similar in how easy they are to work with. The custom needles, as previously mentioned, pierce the skin fairly easily. The Natus needles pierced better than previously used commercial electrodes, but still not as well as the custom needles. The recordings that were collected during this experiment exhibited sinusoidal noise from the environment. Therefore, this experiment was another attempt to further reduce this noise from the signals to clearly detect an action potential.

VI. Invertebrate Leg Test to Compare Signals with Custom Needles

(experiment conducted by Brooke Villinski using the developed protocol)

Crickets are another well-established nerve model, similar to the horseshoe crab used in previous experiments. Due to a shortage of horseshoe crabs at the time of the experiment, crickets were used to test the custom needles compared with the Natus needles. Crickets were used due to simplicity, but also because they are inexpensive and readily available. The cricket as a neurophysiology model works by external sensory structures, called cerci. The cerci are like antennae and can be mechanically stimulated to

produce action potentials. Mechanically stimulating nerves can be done in many ways, some of which include vibrations, pressure, and touch.[36] For the purposes of comparing the custom needles to commercial needles, this factor was useful to not have to rely on the BIOPAC software for stimulation, but also to simplify the experiment's required technology.

Based on the procedure adapted from Dagda et. al., a cricket leg test was conducted to compare the two needles. The recording electrode was inserted in the coxa, and the ground was in the tarsus; these are two of the four segments of the cricket leg. The cricket leg was mechanically stimulated with a blow of air from the experimenter. The experimental setup for the Natus needles, which was similar to the custom needles, is illustrated in Figure 24, where the electrode positions in the cricket leg are shown.

Figure 24. Experimental setup for the cricket leg test with the Natus commercial electrodes pictured. The nerves were stimulated mechanically, with the recording electrode in the Coxa and the ground in the Tarsus. Reproduced from Brooke Villinski.

The overall purpose of this test was to relate the results with the Natus electrodes versus the custom electrodes as a "first pass" to compare recording performance. The results from this experiment, are detailed in Figure 25, where Figure 25A is with the Natus needle and Figure 25B is with the custom needle. The blue line indicates the start of stimulation, which is why there is an increase in signal following it.

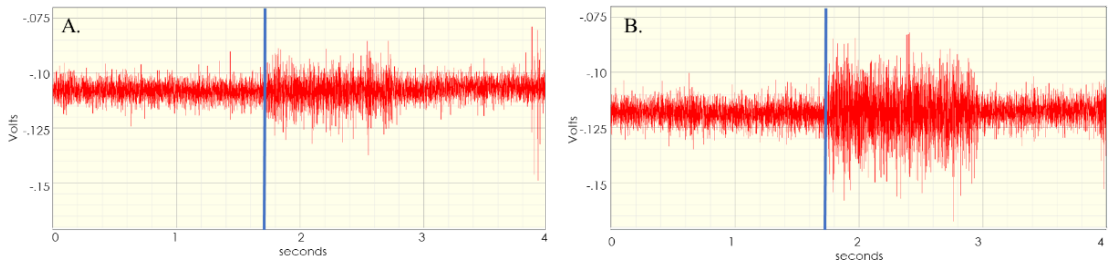

Figure 25. Results collected from the cricket let experiment with A) a Natus electrode, and B) a custom needle. The noise levels are comparable between the two needles. Reproduced from Brooke Villinski.

From this experiment, it can be concluded that the noise levels are comparable between the two, averaging about 12mV for both; this could indicate the needles behave similar in the tissue. For the custom electrode, the response after stimulation is larger. This could be because the custom needles are truly more sensitive than the commercial comparison electrode. However, it should be taken into consideration that the stimulation during this experiment was fairly uncontrolled. The puff of air could have been stronger for the test with the custom electrode, which could have stimulated more nerves and thus increased the resulting signal. As a result, for this test, a good comparison of the recording performance is the signal noise amplitude to show similarities in needle characteristics such as resistance and conductivity. The signal noise amplitudes are comparable between the two needles, which was a first pass to ensure the custom needles work as well or better than commercially available nerve conduction study electrodes. Therefore, more tests with a mouse can be done to further validate the custom needle electrodes given that they are currently not used for nerve studies.

VII. Sciatic Nerve Stimulation in an Anesthetized Mouse

(experiment conducted by Brooke Villinski using the developed protocol)

Before recordings are taken from the scWAT and paw, areas of the mouse that are not widely studied for nerve conductance, well-studied areas were ideal to start with officially comparing the custom needles with commercially available electrodes. In this experiment, the mouse's sciatic nerve was exposed and the custom needles and Natus needles were both used to take recordings using this well-studied nerve being stimulated. Single needles were used as opposed to needles being in an array, and the ground electrode was inserted into the tail. The sciatic nerve was stimulated with a Natus needle for both recording electrode types, and recordings with both the Natus needles and custom needles were taken from the plantar paw with the ground electrode in the tail. The purpose of this experiment, like previous experiments, was to compare recordings in a mouse between the custom needles and well-established needles.

This was the first experiment from an anesthetized mouse that clean action potentials were able to be distinguished from the recordings because the noise level issue had been mitigated. The mouse was stimulated with 50mV, and the amplifier was set to a 200x gain. The stimulation was tested prior to testing the needles in the mouse tissue to ensure stimulation was actually occurring. Figure 26A shows the signal from the Natus needle recordings, and Figure 26B shows the recordings taken with the custom needle electrode. The blue line indicates the time of stimulation, which creates the large increase in signal. The drop in signal shortly after is from the differential amplifier, and the following rise in signal is a compound action potential (CAP), indicated in the figure. CAPs are the summation of multiple different action potentials as it is an extracellular

recording, which makes sense in the context of the recordings taken. The amplitude and shape of signals are very similar between the two electrodes, which was a positive outcome given that the custom electrodes are not typically used for nerve recordings.

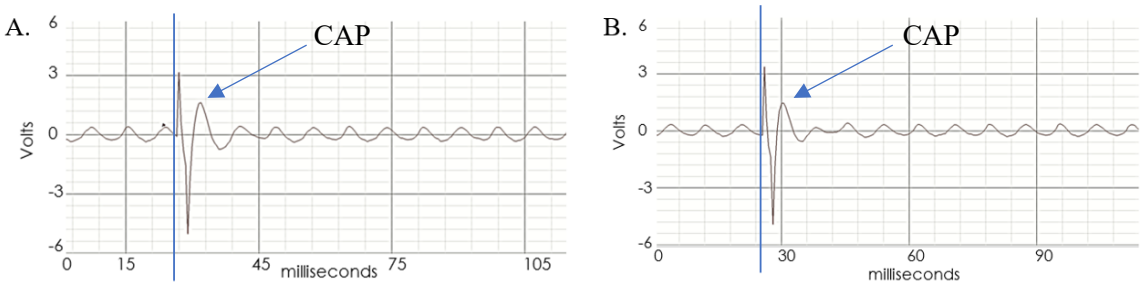

Figure 26. Action potentials recorded from the experiment. A Natus electrode was used for stimulation, indicated with the blue line, and the recording electrodes from A) Natus needles and B) custom needles. The signals are very similar between the two needles. Adapted from Brooke Villinski.

Another recording that was taken from this experiment is testing a custom needle inside an array. The same setup was used with the Natus needle in the sciatic nerve for stimulation, but the recording custom needle electrode was inserted within an array to ensure the array components did not affect signals. Figure 27 is sample data from this experiment, where the blue line again indicates the time of stimulation. A similar CAP is observed that is similar in shape and size to the standalone custom needle in Figure 26B.

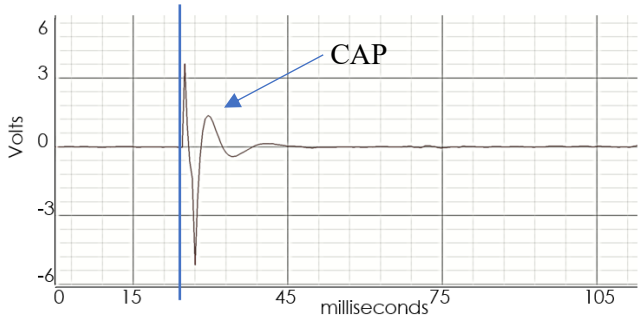

Figure 27. Action potential recorded after stimulation with a custom needle within an array to determine if signals are affected when inserted into the array. The signals of the standalone needle and the custom needle are comparable. Adapted from Brooke Villinski.

Overall, from this experiment, it can be concluded that stimulated recordings can be taken from an anesthetized mouse, and the signals between commercially available needles and the custom needles to be used in the future array are comparable. Additionally, the needle array itself does not affect the quality, shape, or amplitude of signals using the custom needles.

VIII. Stimulation of Anesthetized Mouse with a Functioning Array

(experiment conducted by Brooke Villinski using the developed protocol, array designed and printed by Julia Towne)

The previous experiment tested a working protocol with minimal signal noise to stimulate an anesthetized mouse. The next step in validating the array concept is to test recordings from multiple needles held in the array. A similar prototype that was used in a previous experiment in testing the array was used where nine electrodes are used with a 2 mm needle penetration depth. The needles were arbitrarily numbered, and one electrode was used for stimulation. Recording pairs within the array were grouped to evaluate the quality of signals in an array, as indicated in Figure 28.

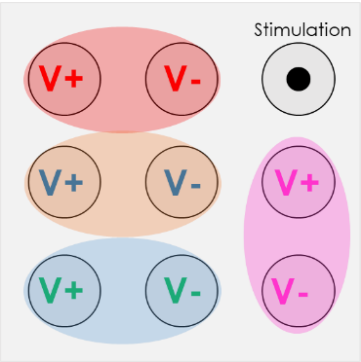

Figure 28. Schematic detailed the recording pairs and the stimulation electrode where differential recordings were taken in the experiment. Reproduced from Brooke Villinski.

Essentially, the goal of this experiment was to record four differential electrode pairs and to stimulate with the ninth needle in an array. The ground electrode was placed in the tail, and the needle array was inserted into the shaved scWAT tissue. A printed circuit board (PCB) was used to wire the electrodes to the amplifier to make handling the array with the mouse easier in future experiments. The PCB with the array in the scWAT of the mouse is illustrated in Figure 29, again with the differential pairs indicated by color.

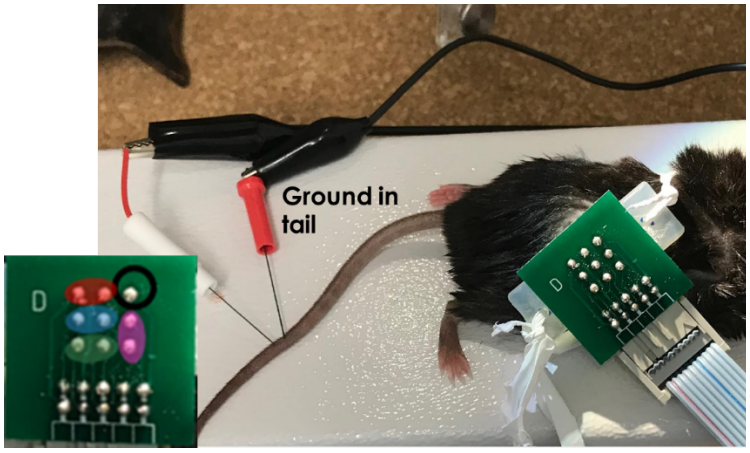

Figure 29. Array setup for the experiment to record from four differential pairs with a PCB board in a needle array. The mouse was anesthetized and recordings taken from each of the color-indicated recording pairs to determine if array recordings are possible. Reproduced from Brooke Villinski.

The results from the experiment are encouraging for pursuing recordings with the selected custom needles. Sample recordings from all differentials are detailed in Figure 30, with the blue line indicating the stimulation time. The large spike followed by the large drop in signal is due to the stimulation and the differential amplifier; however, the increase in signal after are compound action potentials. The blue and pink electrode pairs closer to the stimulation needle, aside for the red pair, show higher signals compared to

the green pair that is further away from the stimulus. This is what one would expect given that needles further away from the stimulation have more distance to lose signal to the surroundings. The green pairs in the array could be too far away from the stimulation needle to yield an action potential from surrounding nerves; the needles in future iterations could be closer together to determine the maximum amount of distance between needles to still get meaningful recordings.

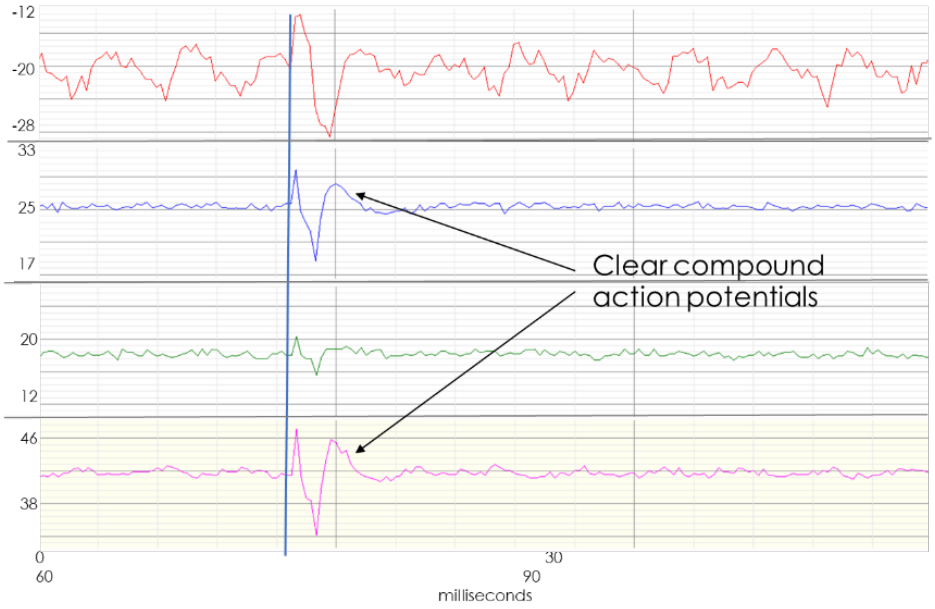

Figure 30. All recording data collected from the needle array experiment. Differential pairs are indicated by color. As the distance from the stimulation electrode increases, the CAP signal decreases, as expected. Reproduced from Brooke Villinski.

There are several different factors that could have affected the results for the red differential pair. The red differential pair was close to the stimulation needle, so it is possible it was along the same nerve bundle. This could have affected the results of not obtaining an action potential by being oversaturated with the stimulation voltage. Additionally, it is possible these electrodes were not fully penetrating the tissue, so the stimulation response from the nerves in this area was not captured. However, despite this

discrepancy of this differential pair, from this experiment, it can be concluded that recording from an array is possible. The overall magnitude of measured compound action potentials decreases the further away from the stimulation needle, as expected, so more experiments can be conducted to further push the progress of a longitudinal study of diseased mice.

Summary of Results

From the series of experiments conducted thus far, several critical conclusions were made. First, much has been learned about BIOPAC and what the final needle array will look like. In terms of needle dimensions, the needles should be put as close together as possible to make the overall array smaller. A rod in the center of the array to apply constant pressure is unnecessary, but the idea of a holder for the needles to sit in is important. If the needle array is simply just a holder for the needles, then the array can be sanitized and used many times simply by just switching out the needles. Additionally, because of the needle holder, a cap to cover the needles is another design aspect that should be considered so the electrodes do not fall out of place. More dimensions of arrays should be tested to further optimize the shape and usability for a final prototype.

Furthermore, protocols and familiarity with new hardware and software had to be established. The BIOPAC software and hardware was selected due to its reproducibility and sensitivity, as well as its broad range of capabilities and features. However, BIOPAC has not necessarily been used for the purpose of recording from small nerve fibers, so limits of sensitivity had to be configured. Detection limits and sensitivity of the amplifiers are important when considering the unknown size of action potentials that will

be collected from the peripheral areas. Understanding how the amplifier and detectors function, as well as the types of signals they collect, will be important downstream in the process to compare nerve signals between mice and between a diabetic and healthy mouse. From this project, working BIOPAC hardware and software protocols have been developed and tested, so both basal and stimulated recordings from mice are possible.

More work needs to be done to establish an optimized design, and many things must be considered when designing the device, including constraints in 3D printing capabilities, dimensions of the needles, the electrical characteristics of the needles themselves, and more. Most of the start of this project has been developing protocols to use the software and hardware to record signals, as well as comparing needles and array dimensions. This mostly includes testing more 3D printed arrays and looking more into how recordings from a needle array will be done. A couple of arrays were tested as the first attempts to do so, but more should be tested to optimize the design. In one experiment, one needle was used as a reference electrode, so this is something that can be incorporated into future array recordings. The distances between needle electrodes will be important for analyzing data and understanding the electrical signals that are collected, too, as the signals change with distance. Overall, before a longitudinal study is conducted, more needle arrays should be tested to verify and optimize the device.

Another aspect of the platform is determining how the data will be used and what measure will be best suited to comparing electrical signals. In nerve conduction studies, typically the nerve conduction velocity and the nerve amplitude voltage are used. These measures could work but incorporating them into the developed system to establish baselines and eliminate noise from the signals will require additional investigation.

Other small portions of the project include tissue staining and imaging familiarity. The Townsend Laboratory had never used the Luxol Blue stain before, so after receiving this stain, part of the project to design this platform was to develop a procedure for staining the tissues to look at myelinated nerves. To do so, several attempts were made with different incubation times and tissue types with the stain to optimize and then image with brightfield microscopy. Furthermore, as the device will eventually develop into a startup company, several Maine Innovation, Research and Technology Accelerator (MIRTA) meetings were attended. These meetings focus on commercialization, understanding the consumer and audience, as well as outreach to establish an effective startup company and device from a business perspective.

Overall, the objective of this project was to start the process of developing a platform for creating a device to detect peripheral neuropathy earlier than it currently is. Many protocols were established and optimized in order to become familiar with hardware and software needed to record electrical signals. Additionally, needle prototypes were tested, and several attempts were done to characterize the needle's properties. Determining an optimal needle and array dimensions are things that are in progress for a final prototype of a design. The work conducted and illustrated in this thesis have provided a way to collect signals, an attempt to understanding the signals, and provided insight into what aspects and constraints will be in the design of the device.

DISCUSSION

There is an increasing need for peripheral neuropathy detection in a clinical setting. The growing obesity epidemic has made nerve death at the limbs an increasing health concern that holds much importance worldwide. The eventual goal of the project is to create a device to ultimately detect peripheral neuropathy sooner than it is currently in the clinic, but this is an iterative process where many things must be considered. The protocols and experiments conducted thus far for designing the platform to do so are just the first steps in the process to create a working device. The design of the device will incorporate a needle array and a longitudinal study of mice with diabetes will assess the quality of this array in comparison with other methods.

The recordings that are typically conducted in both mice and humans are those from large nerves, such as the sciatic and the sural nerve. Not many individuals have attempted to record from small nerve fibers in areas such as the paw, but there is comparable literature for recording in an array in these larger areas of the peripheral nervous system. With standard-of-care devices and techniques, and even typical nerve conduction studies, the designed device will be comparable. Similar recordings and measurements are taken, as particular action potentials are distinguished in recordings after stimulation with the custom needles. Diabetes can be diagnosed with a NCV study with measurements such as the SNAP amplitude given that nerve stimulation induces an action potential of a given magnitude, and such can be compared to a healthy individual.[32,37] As a result, the designed device is comparable as the action potential amplitudes acquired can be compared in a similar fashion to NCV studies, so a diagnosis between healthy and diseased individuals is possible.

In addition, it was proven that muscle tissue can be stimulated and recorded from with the custom needles. Another aspect of the standard of care for diagnosing peripheral neuropathy refers to the CMAP.[37] Compound action potentials were collected in the set of experiments and muscle tissues were target tissues because they are so well understood. Although the neuropathy is far along by the time it reaches the muscles, in the initial stages of the device for proof-of-concept, it is critical to compare the CMAP amplitudes recorded from the custom needles to those taken from commercial electrodes, such as the Natus needles that are used in nerve conduction studies. This comparison would yield similar data, as recording components thus far are comparable to data collected based on the diagnostic standard of care of peripheral neuropathy.

In addition to being comparable to the standard of care for diagnosis, there are researchers working to record from a needle array. Particularly, some of these electrodes that record from peripheral areas are "cuff electrodes"; essentially, there are microneedles or a conductive pad that is encompassed in a cuff that can be wrapped around a wrist, ankle, arm, etc. However, maintaining the positioning of these electrodes is an issue for sustained recordings, and there are sensitivity challenges.[38] Compared to these cuff electrodes, although desirable due to ease of use and ability to customize, the designed device would likely serve a similar function but improve on areas the cuff electrodes fall short in. For example, when using standard NCV study electrodes such as the Natus needles, similar sensitivities and noise levels were recorded between the commercial and custom needles. The Natus needles are more sensitive than cuff electrodes because of their overall purpose, and because the custom needle arrays are comparable to the Natus needles, the custom needle array design improves sensitivity while still being easy to use.

As a result, the custom needle array would be the middle ground between cuff electrodes that are not sensitive enough and the NCV needles that are painful and invasive.

There are a variety of flexible microneedle arrays depending on the fabrication material, and cuff electrodes are just a subset of such. Specifically, flexible microneedle arrays are used for at-home bio-signaling; these signals include other standard of care measurements for diagnosing peripheral neuropathy, like an electromyogram. A benefit of flexible microneedle electrodes not positioned as a cuff is for better electrode positioning and stability. These electrode arrays can be bent in any shape to conform to the subject's skin which enhanced electrode penetration.[39] The designed device thus far is made of rigid 3D printed materials, but making the array flexible is a potential improvement for the future. If the array backing for the custom device is flexible, this could allow for better recordings to be taken given, for instance, the curvature of the foot. A cuff electrode would not be suitable for the tapered shape of the foot, but a flexible electrode would morph to the curvature of the ball of the foot for earlier detection of nerve malfunction.

Another aspect of microneedle electrode arrays that are currently used have a varying needle heights. The Utah Slant Electrode Array features microneedles with a slant to penetrate different depths of tissue, as shown in Figure 31.[40]

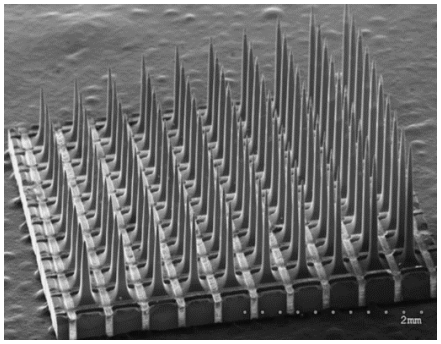

Figure 31. Utah Slant array highlighting the differences in needle heights. Reproduced from Ref [40].

With this slanted electrode setup, peripheral nerves are targeted. However, experiments with this electrode focus on the sciatic nerve and not the areas the custom device will focus on such as the paw and scWAT. In studies with the slanted array, there is stimulation over a time course to track the threshold measurements after insertion.[40] This is comparable to the custom needle array as similar recordings will be taken with stimulation. From these commercial electrode arrays, the potential for different needle heights could be proven beneficial. If the needle array could penetrate the skin layers, the underlying adipose, and then muscle tissue, the progression and severity of neuropathy in the area could be evaluated. Instead of one electrode height where the electrodes penetrate one tissue type, incorporating different needle heights would allow for recordings from each tissue for a more well-rounded diagnosis. Peripheral neuropathy moves from the skin inward, and neuropathy is evident in the adipose tissues.[13] Slanted needle heights in the custom device to penetrate the underlying adipose tissue, as well as other tissue types, would improve the overall quality of the device and understanding of the disease in general.

An important component to that makes microneedle arrays desirable is the ability for these electrodes to be available for at-home use. With an increasing aging population, microneedle arrays will hopefully reduce the need for long-awaited doctors visits, but also reduce preparation time for any gel or lubricants for larger, more invasive needles.[39,41] The designed microneedle array will be easy to use and implement for an individual and be much less invasive. The goal is to make custom design able to fit in the palm of one's hand so it would be portable, lightweight, and efficient. These standards are crucial for microneedle arrays as the small size and scale make these arrays desirable.

To compare in a competitive commercial market, the device must be at least the same size, and if not, improve on weakness areas, such as the addressed sensitivity.

FUTURE DIRECTIONS

Once a working prototype is established, there is a clear direction moving forward on a longitudinal study to track the progression of diabetic mice compared to healthy mice. Young mice will be used, and the disease progression will be tracked using many different diagnostic methods. As previously mentioned, the Townsend Laboratory has established both a genetic and environmental mouse model of mice that will develop peripheral neuropathy. Therefore, starting out, young mice that will develop neuropathy either by genetics or by environmental causes, like a high fat diet, will be compared to healthy mice.

There are several different methods that will be used to track the progression of disease. A Von Frey test is one currently used in the Townsend Laboratory and in the clinical diagnosis of neuropathy. It involves filaments with varying forces that are pressed against the mid-plantar surface of the mouse's paw. The amount of force and the time the animal takes to react to the force is used as a relative measure of neuropathy. The Townsend Laboratory frequently uses this method for neuropathy progression and diabetic studies, so standard and diseased baselines with this test are known. As a result, a Von Frey test will be used in a series of others to track the progression of disease.

Protocols that were established in this work for a cold plate and acetone test are other measures of neuropathy that will be utilized. A cold plate and acetone test are like the Von Frey, where the animal's reaction to each is recorded. Any delayed or unusual responses would be indicative of some extent of nerve death. Each of these behavioral tests—Von Frey, cold plate, and the acetone test—will be used to compare to the

electrical signals obtained from these mice. Ideally, these tests, in conjunction with electrical signals from the developed protocols, would be conducted once a week over the course of several weeks. Additionally, some mice will be euthanized throughout the study and their tissues collected as a small portion of the project was to become familiar with different staining techniques. Luxol Blue is a myelin stain, so this could track the progression of demyelination that occurs with peripheral neuropathy. The myelin that surrounds some nerves will stain blue, and the surrounding tissue will be a clear white, as detailed in Figure 32, which shows an image stained with Luxol Blue from the scWAT.

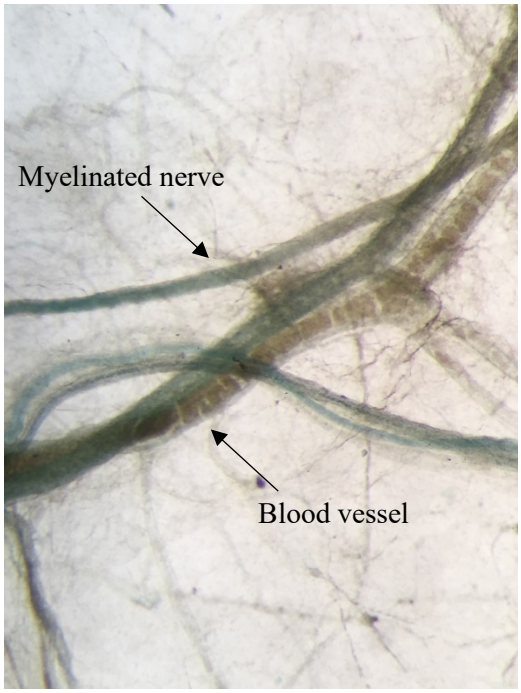

Figure 32. Image from the scWAT tissue of a mouse stained with Luxol Blue. Both blood vessels and myelinated nerves can be distinguished in tissues stained with Luxol Blue.

PGP9.5 is another stain that will be used after tissue collected. PGP9.5 is a pan-neuronal antibody stain, so all nerves in the tissue will be stained, as shown in Figure 33.[42] As a result, nerve death can be compared at different stages of disease with the amount of stain that is imaged. The two stains, PGP9.5 and Luxol Blue will then yield a

full picture at the tissue level of the underlying adipose nerves. The results from the staining can then be compared to the electrical recordings and behavioral tests to give a functional, behavioral, and pathological comparison of peripheral neuropathy.

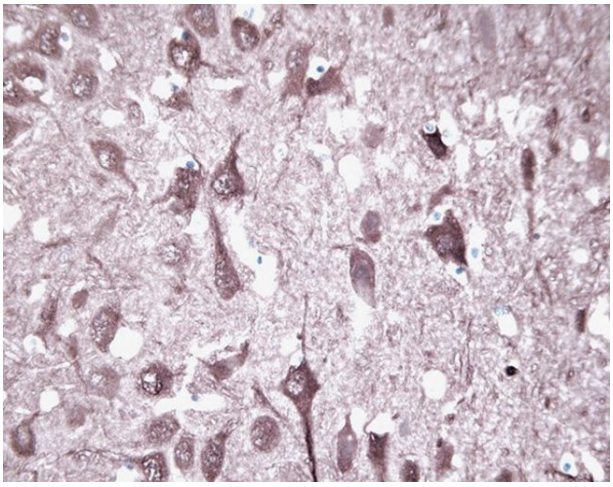

Figure 33. PGP9.5 antibody pan neuronal marker. Reproduced from Ref [42].

In the near future, a longitudinal study will be conducted using mice as a model of disease to identify the sensitivity and quality of the designed device as a diagnostic tool. The work conducted is part of a bigger picture to attempt to diagnose peripheral neuropathy earlier in the clinic by using a less invasive and painful method. In doing so, hopefully the peripheral neuropathy worldwide presence can be better understood, diagnosed, and treated, and diabetic individuals can have a better quality of life.

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APPENDICES

APPENDIX A

IACUC Approval Letter

Office of Research Compliance
Institutional Animal Care and Use Committee

1865 THE UNIVERSITY OF MAINE

311 Alumni Hall
Orono, Maine 04469-5717
Tel: 207-581-2657
www.umaine.edu

September 16, 2019

To Whom It May Concern:

The Institutional Animal Care and Use Committee (IACUC) at the University of Maine has approved the following protocol:

Protocol #: A2017-09-04

Protocol Title: Brain and Whole Body Mechanisms for the Regulation of Energy Balance: Adipose Tissue Neuropathy and Neurotrophic Factors Project

Principal Investigator: Kristy Townsend

Date of Approval: 11/20/2017
Date of Expiration: 11/19/2020

Please contact Paula Portalatin, Research Compliance Officer III, 207-581-2657 or paula.portalatin@maine.edu with any questions.

Best regards,

Paula Portalatin
Research Compliance Officer III

APPENDIX B

BIOPAC Recording Protocol

BIOPAC SOP
EM & JWW
Updated July 23rd, 2019

SECTION I – Set-up and Recording

White cord: stimulating needles, which are connected to black leads
Red alligator hook leads: differential recording needles, labeled with "+1" and "-1"

Software Start-up:

1. Turn on VisionTek external USB connector (back right).
2. Turn on laptop (right) with usual Townsend lab password.
3. Insert teal BioPac USB into VisionTek USB reader.
4. Turn on BioPac on MP160 (in the back on BioPac hardware all the way to the left)
5. When computer is on, open AcqKnowledge icon on desktop.
    a. NOTE: if at this point an error occurs, try bypassing the VisionTek by plugging the black BioPac USB cord and teal BioPac USB directly into the laptop. Restart BioPac and software.
6. Adjust left most amplifier settings (subject to change) - *indicated on amplifier itself

Basal Recordings	Stimulation Recordings
1000x gain	200x gain
3kHz LP	3kHz LP
ON	ON
0.5 Hz HP	0.5 Hz HP

7. Select the "Create new experiment" and then open an existing graph template. Examples of existing templates:
    a. Basal Recordings: "2channelMCE100C_test2"
        i. NOTE: there is no "stimulation" in the name
    b. Stimulation: "2channelMCE100C_Stimulation_0.2Volts_2msec pulse
        i. NOTE: stimulation voltage and length of stimulation pulse can be modified after opening existing graph template – *See Section 2*

Acquiring Recordings:

8. In the Faraday cage, for recordings, open the alligator hook leads and clamp in the desired electrodes
9. Using the manipulator, insert the electrode into the tissue.
10. Insert the other red differential wire with the desired electrode into the sample.
    a. NOTE: the +1 lead and -1 lead labeling does not matter for which electrode is which because the amplifier will just measure. These red leads with +1 and -1 are RECORDING needles, so they will be needed for basal and recording needles.
11. If stimulating, follow the thicker white cable (looks like a phone jack) into the cage from the amplifier. Insert the black stimulation lead labeled "signal," and this electrode is where stimulation will come from.
    a. NOTE: the green wire that extends from the larger white wire is the stimulation ground. Only use if necessary, i.e. signal is noisy and trying to reduce noise by trial and error.
12. Insert the black lead from the cage labeled "ground" into an appropriate position on the sample.
13. Close the Faraday cage if possible. Press "Start" (top left of software) if needles and electrodes are accurately placed.
14. Settings (i.e. high/low pass filters, electrode positioning) can be changed throughout, but the times where things are changed should be recorded. There is a stop bottom where start in the top left was clicked that will stop collection if necessary.
15. Save the data as follows:
    a. <u>Basal recordings</u>
       "INITIALS_date_file description"
       ex. "EM_14dec2019_2 needle recording array dead mouse"
    b. <u>Stimulation</u>
       "INITIALS_date_file description
       ex. "EM_14dec2019_BD needle stimulation dead mouse"

16. After recording and saving data, if conducting another experiment, close out of AcqKnowledge and reopen the GTL template.

Close Out of Software:

17. Close AcqKnowledge icon
18. Eject BioPac teal USB on the laptop and remove from VisionTek.
19. Turn off BioPac MP160 hardware, VisionTek USB reader, and laptop.

SECTION II - Manipulating Software and Tips

Modifying Stimulation and Pulse:

1. Select **MP160→Set up data acquisition→stimulation** (top left of software)
2. Change the voltages and the timing in between intervals as needed.
    a. NOTE: for changes to be applied the run must be stopped and started again
    b. Below are two examples of settings

Segment #	Voltage (V)	Time (ms)
1	0	100
2	0.2	2
3	0	100
4	0.2	2
5	0	100

Example of dual 0.2 V, 2 ms pulses with alternating 100 ms and 200 ms delays. This is what was being used previously.

Segment #	Voltage (V)	Time (ms)
1	.05	2
2	0	300
3	0	0
4	0	0
5	0	0

Example of what yielded positive results for sciatic nerve in mouse. A single 0.05 V, 2 ms pulse followed by a 300 ms delay.

3. Save as template with a new name with the given settings that were changed.
    a. For example, include the stimulation and voltage magnitude in the name, i.e. "2channelMCE100C_Stimulation_0.05volts_2msec pulse"

Modifying Scales:

1. Adjusting Voltage Scale (**y-axis**)
    a. Double-click y-axis
    b. Popup screen will appear.
    c. Adjust scale anywhere from **1-15 V** depending on size of signal and or noise.
2. Adjusting Time Scale (**x-axis)**
    a. Double-click y-axis
    b. Popup screen will appear
    c. Adjust time to **200ms** to distinguish wave

APPENDIX C

COLD PLATE PROTOCOL

Cold Plate Test Protocol (Thermal Allodynia)
Adapted from Van der Waal et al. 2015
EM Updated February 2019

1. Cover the bottom of the Von Frey apparatus with aluminum foil and tape to the sides of the apparatus. Ensure the mouse's feet can still be seen past the tape and aluminum foil.
2. Determine the number of experimental subjects and place each mouse into its own area within the Von Frey apparatus. Allow acclimation for 15-20 minutes.
    a. NOTE: Make sure to properly label each compartment with the necessary mouse information (i.e. any identifying markers: mouse ID, ear punch, sex, etc.)
3. Place the entire Von Frey enclosure into a bucket containing ice with a thin layer of aluminum foil across the top of the ice and record the time.
4. Using a stopwatch, observe and record the time at which any of the potential reactions listed below occur, being sure to record any corresponding intensities for each reaction:
    a. Lifting of the paw or on hind legs
    b. Scratching of the paw
    c. Touching side of hip or paw depending on side of paw administration
5. Record the time that the cold plate reaches 2-2.5 ºC.
6. Record the amount and intensity of each behaviors exhibited over a period of 4 minutes.

Cold Plate Test Data Recording Template

Cold Plate Neuropathy Test Template Date: ___________________

Start Time: End Time:

People participating: Project/Cohort: _______________________

Mouse ID	Time started in cold	Time when 2-2.5°C reached	Lifting of paw/on two feet	Scratching	Touching side of hip or paw	Total Latency Behavior Time		
						Lifting	Scratching	Touching
			0:05 0:10 0:15 0:20 0:25 0:30 0:35 0:40 0:45 0:50 0:55 1 1:05 1:10 1:15 1:20 1:25 1:30 1:35 1:40 1:45 1:50 1:55 2 2:05 2:10 2:15 2:20 2:25 2:30 2:35 2:40 2:45 2:50 2:55 3 3:05 3:10 3:15 3:20 3:25 3:30 3:35 3:40 3:45 3:50 3:55 4					
			0:05 0:10 0:15 0:20 0:25 0:30 0:35 0:40 0:45 0:50 0:55 1 1:05 1:10 1:15 1:20 1:25 1:30 1:35 1:40 1:45 1:50 1:55 2 2:05 2:10 2:15 2:20 2:25 2:30 2:35 2:40 2:45 2:50 2:55 3 3:05 3:10 3:15 3:20 3:25 3:30 3:35 3:40 3:45 3:50 3:55 4					
			0:05 0:10 0:15 0:20 0:25 0:30 0:35 0:40 0:45 0:50 0:55 1 1:05 1:10 1:15 1:20 1:25 1:30 1:35 1:40 1:45 1:50 1:55 2 2:05 2:10 2:15 2:20 2:25 2:30 2:35 2:40 2:45 2:50 2:55 3 3:05 3:10 3:15 3:20 3:25 3:30 3:35 3:40 3:45 3:50 3:55 4					
			0:05 0:10 0:15 0:20 0:25 0:30 0:35 0:40 0:45 0:50 0:55 1 1:05 1:10 1:15 1:20 1:25 1:30 1:35 1:40 1:45 1:50 1:55 2 2:05 2:10 2:15 2:20 2:25 2:30 2:35 2:40 2:45 2:50 2:55 3 3:05 3:10 3:15 3:20 3:25 3:30 3:35 3:40 3:45 3:50 3:55 4					

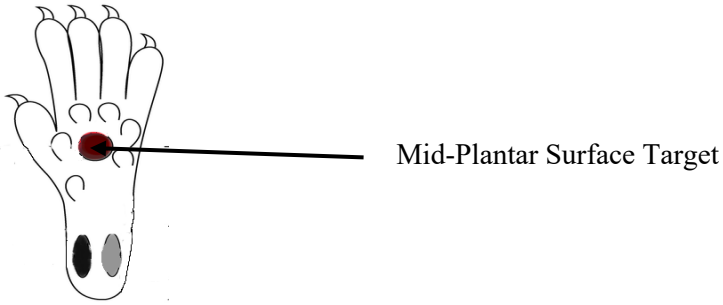
Observations:

Figure 34. *Cold plate test data recording template. There are areas for specific information about the study, such as the mouse ID and project. The time when the mouse was put on the cold surface is recorded, and the time when the cold plate reaches the proper temperature. There are time boxes for the lifting of paw/on two feet behavior that can be shaded accordingly when the mouse performs that behavior. Other times for other less frequent behaviors can be recorded, as well as any observations. For analyzing the data, the total latency behavior time is used.*

Acetone Touch Test Protocol

Neuropathy Acetone Test
Adapted from Vissers, et. Al (2005)
EM Updated February 2019

1. Determine number of experimental subjects and place each mouse into its own area within the Von Frey apparatus
    a. NOTE: Make sure to properly label each compartment with the necessary mouse information (i.e. any identifying markers: Mouse ID, Ear Punch, Sex, etc.)
2. A total of 12 mice may be tested at once, but be sure to allow each mouse approximately 15-20 minutes to acclimate to the mesh flooring before testing
    a. NOTE: Mice have acclimated when excessive movement and grooming have ceased.
3. Set up video camera so the mice can be seen
4. Fill a 1 mL Tuberculin syringe with acetone and gently push the syringe until a small bubble forms at the top of the syringe
5. Start recording on the camera. Gently apply the acetone to the plantar surface of the hind paw (see diagram below), making sure to only touch the mouse with the acetone bubble formed at the top of the syringe.



6. Observe each mouse for any of the following acetone evoked behaviors:
    a. Paw elevation
    b. Flinching
    c. Licking
    d. Scratching
7. Using the video camera, record how long these acetone evoked behaviors are present for a minimum of 0.5 seconds (for the brief normal response) to a maximal cut off of 20 seconds (Choi et al. 1994)
    a. NOTE: Time 0 begins when the acetone is applied and stops when the behaviors have ceased

AUTHOR'S BIOGRAPHY

Erin Merchant grew up in Windham Maine with her parents. Although being an only child, she has been surrounded by family coming and visiting at her house on Little Sebago Lake. She loves the outdoors, especially hiking, skiing, and boating, all with her golden retriever best friend Zoey. She attended the University of Maine with a major in Biomedical Engineering and was passionate about finishing her honors thesis as an engineering student. Throughout her time at the University of Maine, Erin has had opportunities to work at both Jackson Laboratories and IDEXX as an intern. She hopes to get her M.B.A. in the future after working a couple of years post-graduation in industry.