Stimulation and Recording of Compound Action Potentials in *Lumbricus terrestris*, *Homarus Americanus* and *Cambarus bartonii* nerve cords

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***Abstract*— When an individual suffers nerve damage as a result of a stroke or severe trauma, their quality of life suffers greatly. In many cases, this can lead to paralysis of limbs due to peripheral nerve damage. To improve mobility for these patients, it is necessary to understand the extent to which they are impaired and to remedy it through specialized therapies, such as neurostimulation. Current research in the field of neurostimulation of model organisms, such as earthworms, lobster and crayfish, have demonstrated that it is possible to stimulate nerve bundles to propagate compound action potentials [12,15]. Once these action potentials are sent along nerves, it is possible to stimulate muscle contraction and with enough activation a patient could experience improved limb mobility over time. Ultrasound is a technique that has been widely used to stimulate and record neural activity [15]. The goal of this study is to investigate the feasibility of ultrasound as a method of producing consistent activation of nerves. This project will make use of ultrasound to observe changes that occur in the nerves of crayfish when a signal is being sent through but will also be coupled with electrical stimulation as a proof of nerve viability. Cross-sectional ultrasound images of the nerve will be taken at specific points to observe change in diameter as the action potential passes along the nerve. The diameter is expected to increase and decrease due to the flux of sodium ions [9]. Due to challenges difficulties on obtaining crayfish specimens and technical issues with the electrophysiological equipment, the intended goal of this study was not met. The protocols for an effective study were investigated however and will be incorporated into the experimental framework going forward.**

***Index Terms*—Deep brain stimulation (DBS), Conduction velocity, Neurostimulation, Ultrasound stimulation**

I. INTRODUCTION

Nerve damage that arises as a result of severe trauma or strokes, can seriously hinder a person's quality of life. During a stroke, the blood supply to the brain is disrupted and therefore, oxygen and nutrients are not transported to the brain. In cases such as this limb paralysis is a common side effect damage to peripheral nerves. In the United States, a 2013 study found that strokes were the primary cause of paralysis among 33.7% of people in the surveyed population [8]. This represents a significant portion of the population and therefore techniques to improve mobility are imperative. In order to counter the effects of paralysis, it is necessary to either replace dead nerve tissue or stimulate the remaining live nerves in the area of interest to propagate action potentials through neurostimulation techniques. Replacing damaged nerves in stroke patients is a high risk procedure, so the development of non-invasive neurostimulation methods has increased in recent years. Research in the field of neurostimulation of model organisms such as lobster and earthworms have been able to demonstrate that it is possible to stimulate nerve bundles to propagate compound action potentials.

Additionally, ultrasound is a technique that has also been widely used to stimulate as well as record neural activity [15]. Therefore, applying

ultrasound to the current model organisms could help to validate neurostimulation techniques and provide another method of treatment for stroke patients. While the primary focus of neurostimulation research has been the central nervous system, less is understood about the effects of stimulation on peripheral nerves. Should the stimulation of nerves in a model organism be successful, it is possible that ultrasound techniques could be applied in-vivo to peripheral nerves in human subjects. If the nerves in a patients arm for example were subjected to ultrasound stimulation, action potentials could be propagated along the nerve. This has the potential to stimulate muscle contraction and with enough stimulation it is possible that a patient could experience improved arm mobility over time. While this effect could be accomplished with electrical stimulation of muscle, this technique may cause the development of a rash or burn from electrode contact and is invasive in some cases. Additionally, those with devices like pacemakers could experience interference if an electrical pulse was applied. Being able to perform the same task with ultrasound neurostimulation would mitigate these risks due to the lack of direct contact with the skin.

This project attempted to make use of ultrasound to record the changes that occur in the central nerve cords of crayfish when a signal was being sent through it. However, due to challenges with the electrical stimulation set-up and difficulty in obtaining crayfish specimens, data was not collected for this specimen type. In the past semester, ultrasound images of earthworm nerve cords were taken, so the methodologies behind this process will be expanded upon and will later be implemented into the experimental framework. Additionally, since lobster is similar to crayfish from a physiological standpoint, this will be used as the model for testing the viability of this proposed system with a functional pulse stimulator borrowed from another laboratory.

Ultrasound imaging involves sending megahertz frequency sound waves through biological tissues [1]. The subsequent echoes are detected by the transducer and processed into images which can be used to diagnose and treat various conditions. In addition to imaging, ultrasound can also be employed in modulating brain activity, which has gained increased attention in the medical field [6].

There have been multiple studies that have applied ultrasound to brain stimulation techniques, including low intensity focused ultrasound (LIFU), for the treatment of neurological disorders, and in modifying damaged brain circuits and other biological mechanisms [1]. Additionally, ultrasound has been applied to many medical applications, among which drug delivery and tissue ablation are emerging areas in the medicine [1]. While the focus in research is mainly on ultrasound-induced brain stimulation, for conditions such as Parkinson's, there is potential for applying ultrasound to the stimulation of nerves in the peripheral nervous system and within spinal cord [6]. Ultrasound could potentially be utilized as a form of therapy for peripheral nerve damage [2].

While many models of nerve cords have been developed, the basic assumptions behind the Hodgkin-Huxley model were the basis for this study. This model demonstrates the nonlinear behavior of ion channels in the membranes of nerves. It assumes that there is a higher concentration of sodium ions on the exterior of the neuron [1]. Additionally, these ions can travel through the semipermeable membrane through voltage gated ion channels [10]. When an external voltage is applied to a neuron the voltage gated sodium channels will open allowing the sodium to travel to the interior of the neuron. This in turn increases the membrane potential within the neuron. Should the threshold potential be reached, approximately -55mV, a depolarization event will occur and allow for the generation of an action potential [10].

This concept, based on the assumptions of the Hodgkin-Huxley model, is the basis for postulating that the nerve will experience a change in diameter. As more ions travel into the neuron, the interior becomes more hypertonic. As a result of this change in concentration, water will travel into the cell by osmosis. This would cause the neuron to experience swelling as the action potential propagates down the axon. In is assumed that the depolarization of the axon should be correlated with the change in diameter. Therefore, the observance of an increase the diameter of the nerve could be considered indicative of compound action potentials propagating along the nerve fiber.

Prior to incorporating ultrasound into stimulation and recording of the lobster nerves, consistent electrophysiological recordings have to be produced. Electrophysiological stimulation and recording have been shown to produce compound action potentials [12]. Therefore, when combined with ultrasound recording, images can be generated prior to and during electrical stimulation and provide insight as to the best location to later apply ultrasound stimulation. These images can then be compared to determine whether or not stimulation caused a significant change in the diameter of the nerve cord. Specifically, the ventral nerve cord diameter would be determined prior to and during stimulation. As stated previously a larger diameter is expected during electrical stimulation due to the influx of sodium ions and water as a result of depolarization of the axon. The calculation of the conduction velocity for each trial will be used as a proxy for determining whether the nerve produced compound action potentials.

In future experiments, ultrasound imaging will be utilized to observe these changes in the diameter of lobster nerve cords. In applying external voltage, via ultrasound and the repaired pulse simulator, to the ventral nerve cord, it is expected that compound action potentials would be produced.

II. Materials and Methods

During the course of this experiment, electrical stimulation and recording will be performed on two different lobsters in order to provide a proof of concept of the set-up and to determine the optimal location for stimulation. Prior to this stimulation, each lobster was anesthetized for thirty to forty-five minutes by being submerged in ice. While some limb movement can be detected after this time period, the animal is still able to be dissected for nerve cord exposure. In order to do this the claws of the lobster are removed and placed back on ice. Using a scalpel, the tail is then removed from the rest of the body. The swimmerets, which aid in locomotion, are then cut off with surgical scissors. At this point the exoskeleton will be cut along each side and the connective tissue pulled back and sliced slowly to ensure that the ventral nerve cord is kept intact. The nerve cord is the same color as the surrounding tissue, so a glass rod is used to help distinguish the nerve cord from the tissue. When the tail flexes that is indicative of the nerve being active. Once the nerve is exposed, electrical stimulation can be applied.

Note that for the first trials, the nerve was completely removed from the lobster and placed in a specially designed chamber that I designed in Inventor that features steel electrodes at specific locations [Figure 1]. These trials did not produce definitive results and therefore, it was decided to leave the nerve intact within the tail, in order to determine whether it was still viable. The remainder of this section describes the intact nerve procedure that will later be applied to crayfish.

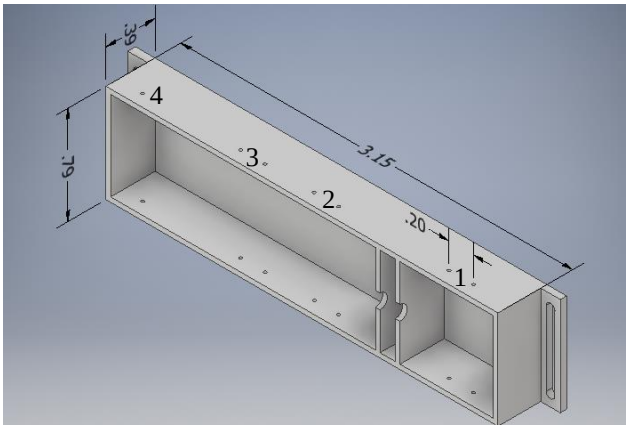

Figure 1. Lobster nerve chamber. Steel electrodes were inserted into each hole from one side of the chamber to the other. It was filled with saline and the nerve was draped over the indentation in the middle wall. Stimulation was applied at position 1 and recording was done at positions 2 and 3. Position 4 was used for grounding. All dimensions are in inches.

The lobster nerve was kept moist by applying a saline solution. This solution consisted of approximately 6.75 g of NaCl, 186.4 mg of KCl, 693.5 mg of $CaCl_2$, 190.15 mg of $MgCl_2$, 2.5 mL of 1M HEPES buffer, 495.55 mg of glucose [16]. These salts and compounds were dissolved in 500 mL of water and then placed on a stir plate for 15 minutes to allow the components to dissolve and adequately mix [16]. In the future, when crayfish are available, a similar saline solution featuring 195mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.6

mM $MgCl_2$, and 10 mM HEPES will be produced in a similar manner [15]. With the crayfish solution however, the pH will be adjusted with NaOH such that it is 7.4 [15]. Nerves must stay moist in order to remain viable, so care will be taken to ensure they are adequately damp throughout experimentation.

For electrical stimulation, electrodes composed of copper wire and/or a steel needle, will be placed in the anterior portion of the nerve, closer where the head was located. The silver chloride recording electrode will be placed at the posterior end of the tail and connected to a Neuroprobe amplifier. To ground the system, an electrode will be within the muscle itself. A biphasic stimulation has been attempted in the past, although this did not produce the desired compound action potential when the recorded signal was passed through a Neuroprobe amplifier and a Butterworth filter to display on the oscilloscope. Therefore, monophasic stimulation will performed for the remainder of the testing. This means that the cathode and anode stimulating electrodes will separated by a distance of approximately 2-4 cm during each trial. The cathode will be inserted closer to the recording electrode and its location is varied along the length of the nerve. These electrodes were just secured with tape and so they were not in place as securely as the recording electrode which attached to a micromanipulator. The distance between the stimulating and recording electrodes were used as the basis for calculating the conduction velocity of the impulse.

The electrical set-up is represented in figure 2 and consists of [1] AM Systems Isolated Pulse Stimulator (Model 2100), [2] AM Systems Neuroprobe Amplifier (Model 1600), [3] Krohn-Hits Butterworth/Bessel Multi-Channel Filter (Model 3944) and a [4] Lecroy Waverunner Oscilloscope (Model LT224). Once the pulse stimulator sent a pulse through the stimulating electrodes, it was picked up by the signal from the recording electrode. From there the signal passed through the neuroprobe amplifier which amplified it by 10x (20 dB) and was passed to the Butterworth filter which served as a bandpass filter for this study (100Hz-6000Hz). Finally, the filtered signal will be displayed on channel 2 of the oscilloscope. Channel 1 on the oscilloscope will display the trigger pulse from the pulse stimulator. The gains on the input and output signals for the Butterworth filter will be manipulated between 0 and 20 dB in order to reduce noise. The waveforms will then be captured with a LabVIEW program (ContinuousOscolloscope_2ChannelsLobeterNerve.vi) which saved the time and voltage amplitudes of the data into excel files for further processing in MATLAB (Mathworks, Natick MA). This particular program collects 500 samples per data capture.

Another electrical stimulation method that will be tested on the crayfish model will be through the use of a suction electrode [17]. In this case the diameter of the nerve cord will be determined. The electrode that is usually reserved for recording, which features a connection to a head stage, will instead be fitted with tubing. This tubing will surround the silver chloride wire of the electrode. A syringe fitted with a specially designed pipette tip tubing apparatus I designed, will be attached to the side of the electrode. The suction applied, in theory, should pull the nerve cord into the tubing and form a solid connection between it and the silver chloride wire. Depending on the amount of pressure the suction electrode provides, a vacuum may need to be applied as well. The apparatus for this stimulation type is still being developed but is expected to be useful in confirming whether stimulation is effective.

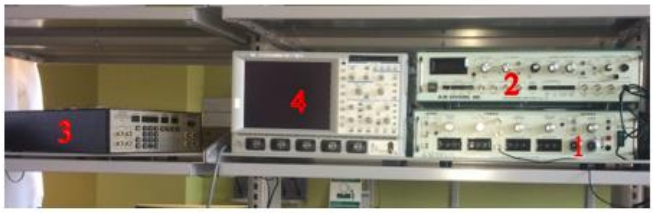

Figure 2. Electrical set-up

For the ultrasound recording, the 30MHz transducer will be mounted above the nerve with a coupling cone I previously designed. It will then be connected to the T/R port on the Olympus Pulser/Receiver (Model 5073PR). The motor that controls the movement of the transducer along the x-axis (Brushed Motor Controller-red) will be

connected to the external trigger in port of the pulser/receiver. This allows it to control the functions of the pulser/receiver when set to external trigger mode. The sync out port and the RF port will be connected to channels 1 and 2, respectively, of the 12-bit oscilloscope (Teledyne Lecroy: HDO4024A). The sync out controls the trigger of the oscilloscope, while the ultrasound signal obtained from the lobster will be displayed on channel 2. The LabVIEW (National Instruments: Austin, Texas) program (RasterScan.vi), will be used to capture the data necessary to reconstruct the image. Once the oscilloscope is switched to sequence mode the oscilloscope can be triggered externally. The LabVIEW program (RasterScan.vi) scanning parameters will manipulated such that there will be a different number of cross-sectional images taken along the y-axis for each of the specimens imaged successfully (1-10 passes in this case). The approximate diameter of the lobster will be measured and used to set the parameters for the x-axis. The transducer with the coupling cone will positioned so that it was between 0.5 mm and 1 mm from the surface of the nerve, but still in contact with the coupling gel.

This procedure has been performed on earthworms as well, although the definite features of the nerve anatomy are difficult to characterize. The same is expected to be the case for imaging lobster and crayfish nerves as well. This could have been due to possible movement of the specimen as the transducer moved along the surface. The images generated when the transducer did not have the coupling cone attached were able to produce images in which the earthworm was visible. In this case the worm was placed in a glass dish filled with the saline solution described above. The transducer was positioned so that the signal obtained on the 12 bit oscilloscope was located starting at 16 micro-seconds. This is the time that the reflected earthworm signal should have been arriving back at the transducer. It will be more difficult to fully submerge the lobsters and crayfish and still stimulate electrically, so imaging without a coupling cone will not be undertaken for these biological models.

For ultrasound stimulation of lobster and crayfish, a single element transducer will be used with a coupling cone [6]. The 30 MHz transducer will be used to accomplish this task. Using the position information obtained from the electrical stimulation set up, the transducer will be mounted above the nerve at that same location. The pulser/receiver will still be used to control the impulses that are sent. The stimulating transducer will remain in a fixed position as opposed to recording transducer which will be in motion during the course of stimulation. A MATLAB script was defined previously, which controls the stimulation parameters. These parameters include the pulse duration, the amplitude, and the frequency of repetition. Due to the unpredictability of ultrasound stimulation, it is expected that multiple trials will need to be conducted in order to achieve adequate stimulation [15].

III. Results

During the course of this experiment, electrical stimulation of the earthworm and lobster nerves was achieved, as demonstrated in figures 3 and 4 respectively. However, transitioning to ultrasound simulation was not successful. In order to accurately record lobster and crayfish nerves in conjunction with the electrical set up, the ultrasound transducer must be fitted with a coupling cone. The coupling cone serves to funnel the ultrasonic waves to a specific position along the nerve. The current design of the cone requires modification to achieve adequate images. The new design should be one millimeter shorter in length so the focal distance should be less. Care needs to be taken in the development of this new cone in Inventor to ensure that the inner dimensions do not interfere with the ultrasound beam.

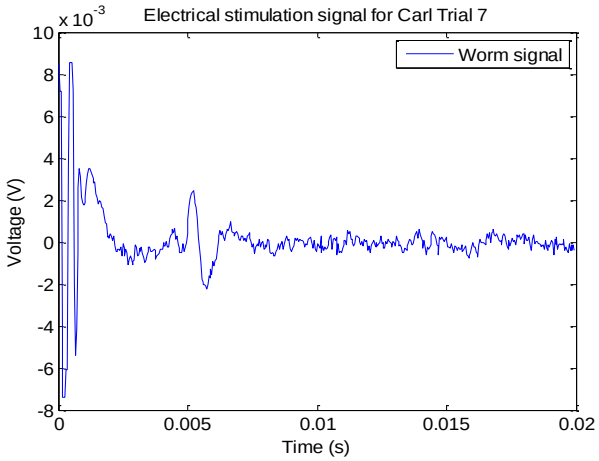

Figure 3. Signal obtained from the worm "Carl". Signal parameters: 2V stimulation pulse, distance between stimulation and recording electrodes 8 cm, 200 μs pulse width, monopolar stimulation. Averaged over 6000 samples.

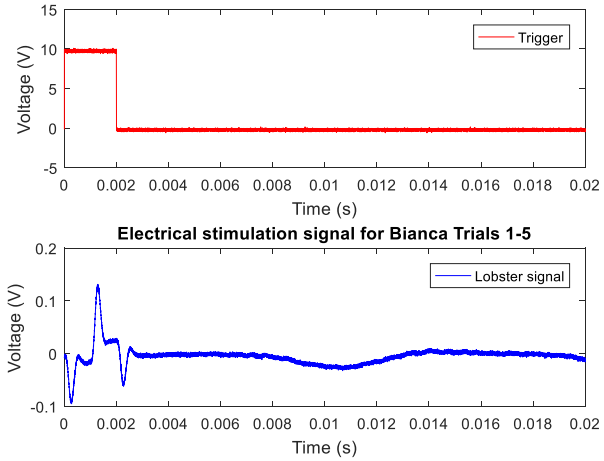

Figure 4. Signal obtained from the lobster "Bianca". Signal parameters: 1 mA pulse amplitude, distance between stimulation and recording electrodes 5.8 cm, 1 ms pulse width, monopolar stimulation. Averaged over 5 trials each containing 50,000 samples.

As shown in figure 3, the peak in the worm signal occurred at 0.002439 volts which happened at 0.005218 seconds after the stimulation artifact. This means that the signal arrives at back at the transducer at the base of where this peak begins. This would occur at the time associated with 0.1 times the voltage of the peak, so approximately 0.0002439 volts. In the data, the closest value to this voltage was 0.0003863 volts, and the time was 0.004978 seconds. Using the distance between the stimulation and recording electrodes, 8cm (0.08m) and this time value the conduction velocity was determined to be 16.1 m/s. The average conduction velocity in earthworm nerves is 12.6-32.2 m/s, so since this value is within this range it can be accepted as a compound action potential [12]. This same process was followed for the other earthworm experiments, but the peaks in these trials were not distinguishable from noise for experiments after the "Iggy" trials.

For the lobster trials it is more difficult to distinguish a definite peak in the signal. There appears to be a depression in the at approximately 0.011 seconds, however it is not certain whether or not this corresponds to a compound action potential. The conduction velocity in this case cannot be determined for these trials. Other experiments have demonstrated that the conduction velocity within lobster medial axon should be between 2.1-10.2 m/s [18]. Therefore, if peaks appear in future trials and the calculated conduction velocity is within this range, that can be considered an effective stimulation which would correspond to a compound action potential.

In terms of imaging, only cross-sectional images of the earthworm have been taken. As shown in figure 5, the distinct features of the worm anatomy are not clearly identifiable. Improvements in the resolution of the images would allow for recognition of the major organs and allow for a comparison of the diameter of the nerve cord prior to and during a compound action potential.

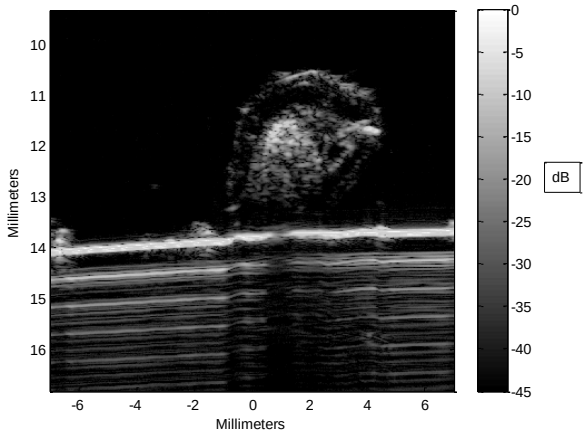

Figure 5. Cross-sectional image of earthworm with no electrical stimulation being applied, no coupling cone attached to 30 MHz transducer.

IV. Discussion and Summary

The electrical stimulation experiments that were performed on the earthworm nerve cords did not yield consistent results each time. The same is true of the lobster electrical stimulation experiments. Electrical stimulation in this case was being used as a means of determining if the nerve was functional in terms of sending a signal. However, since the position of the stimulating electrodes relative to the medial and lateral axons of the nerve most likely changed over the course of data collection, a compound action potential was not seen for all trials. There also could have been interference from the other electrical equipment in the room. Of the trials that did produce a signal after the stimulation artifact, the worm experiment Carl produced the most definitive signal. From this data, plotted in MATLAB, the location of the peak in terms of time was calculated and the conduction velocity was determined. In the case of the Carl trial 7, the trial that most closely resembled the shape of a compound action potential, the average conduction velocity was 16.1 m/s. Although this is within the range specified by Gonzalez et. al., the fact that it is not 12.6 m/s or 32.2 m/s could be indicative of the stimulating and/or recording electrodes being located between the medial and lateral portions of the nerve bundle [12].

Ideally the stimulation from the pulse generator should be done simultaneously with the ultrasound recording; however, this was not achieved due to complications with the electrical stimulation set up, in particular the broken pulse stimulator. It continued to emit a current even though no pulse was being sent. As a result of this bubbles appeared within some of the lobster and earthworm trials. Simultaneous electrical stimulation and ultrasound recording would have ensured that the transducer was recording any changes in the diameter of the nerve cord as the pulse was traveling through it. In order for ultrasound images to be interpreted successful, there needs to be a better understanding of the internal anatomy of the earthworm, lobster and crayfish models. Although the nerve cord is located on the ventral side of the worm, the depth at which the nerve is located was not documented in the literature reviewed for this study. Due to dissection, knowing the depth of the nerve in lobster and crayfish is not necessary, however the dimensions of the nerve cords need to be known.

The images obtained from the earthworm revealed the outlines of the worm, but the resolution does not allow for a definitive characterization of the nerve cord. In this study, a 30 MHz transducer was used, so the use of a transducer with a greater frequency would be capable of resolving more of the fine features of the nerve cord. However, since transducers are expensive, it will be necessary to modify the methodology of the current set up. For each image taken the earthworm was oriented such that the ventral portion was facing the transducer. Therefore, the nerve should be located toward the top of the image in figure 4, between 11 and 12 millimeters from the ultrasound transducer.

There were also challenges with the computer used for data collection. Since it was running on a Windows 7 platform, the applications were not running efficiently. For example, the LabVIEW program (RasterScan.vi) was intended to change the oscilloscope settings to sequence mode. But due to the lag of the system, this did not happen. Therefore, the oscilloscope had to be changed to sequence mode manually before the image could be obtained.

In terms of understanding the biomedical issue of improving mobility for stroke patients, it is understood that stimulation using ultrasound requires more research. In order for ultrasound to be implemented on a clinical scale, the change in diameter of the nerve cord needs to be characterized more precisely. Should the technique of ultrasound stimulation be successful in a modeled system like this, then similar techniques can be applied to human subjects. Ultrasound stimulation has the potential to be an effective method of muscle stimulation. However, verification needs to be found through additional testing on model organisms, like the earthworm, lobster and crayfish. Successful stimulation of nerve cords in this manner would offer an alternative to traditional electrical stimulation, which in many cases can cause skin irritation or be an invasive procedure.

V. References

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