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Characterizing Rat PNS Electrophysiological Response to Electrical Stimulation Using *in vitro* Chip-Based Human Investigational Platform (*i*CHIP)

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ABSTRACT

Ex vivo systems and organ-on-a-chip technology offer an unprecedented approach to modeling the inner workings of the human body. The ultimate goal of LLNL's *in vitro* Chip-based Human Investigational Platform (*i*CHIP) is to integrate multiple organ tissue cultures using microfluidic channels, multi-electrode arrays (MEA), and other biosensors in order to effectively simulate and study the responses and interactions of the major organs to chemical and physical stimulation. In this study, we focused on the peripheral nervous system (PNS) component of the iCHIP system. Specifically we sought to expound on prior research investigating the electrophysiological response of rat dorsal root ganglion cells (rDRGs) to chemical exposures, such as capsaicin. Our aim was to establish a protocol for electrical stimulation using the *i*CHIP device that would reliably elicit a characteristic response in rDRGs. By varying the parameters for both the stimulation properties – amplitude, phase width, phase shape, and stimulation/ return configuration – and the culture conditions – day *in vitro* and neural cell types - we were able to make several key observations and uncover a potential convention with a minimal number of devices tested. Future work will seek to establish a standard protocol for human DRGs in the *i*CHIP which will afford a portable, rapid method for determining the effects of toxins and novel therapeutics on the PNS.

INTRODUCTION

With the cost of drug development consistently on the rise, major efforts are being devoted to developing platforms that more accurately model human physiology. As of November 2014, the Tufts Center for the Study of Drug Development reported that the cost of getting a drug approved by the U.S. Food and Drug Administration and conducting post-market surveillance brings the total life-cycle cost for a pharmaceutical to $2.9 billion.[1] It is well established in the industry that of 5,000 drugs tested one will receive approval by the FDA.[2] According to industry standards, the risks mitigated by preclinical trials is around 10%. Of 250 promising compounds from preclinical trials, only 5 will make it through the first phase of clinical trials. This dramatic attrition as a potential pharmaceutical makes its way through the drug development process into clinical trials is largely due to the fact that animal models, no matter how closely related, are not adequate enough to imitate the human body. In the case of cerebrovascular diseases, particularly stroke, this has led to the oft stated fatalistic proclamation that "everything works in animals but nothing works in people."[3]

In recent decades, a great deal of interest has gone into the development of organ-on-a-chip technologies that utilize *ex vivo* transplants from human tissue to effectively simulate the physiology of the organ being studied. These microengineered tissue systems provide a novel platform for drug screening and toxicology applications.[4] Lawrence Livermore National Laboratory has taken this idea of isolated systems to the ultimate conclusion and seeks to create a full simulation of a human-on-a-chip.[5] By using microfluidic channels, multi-electrode arrays (MEA), and other biosensors, the major organ components can be integrated in such a way that interactions of the whole system can be reliably studied. En route to this goal, the first step is to perfect each of the individual systems.

In this study, the focus of the research and device testing was on the peripheral nervous system (PNS) component. The PNS processes all sensory inputs as well as pain before passing it on to the central nervous system (CNS), making it of major importance for the simulation of the human body. Using the PNS-on-a-chip technology, studies could be conducted to develop novel therapeutics for PNS neuropathies, pain management, or other indications, without requiring injection into a living human.

Prior research using the MEAs and PNS cultures has mostly focused on chemical stimulation, however, there are many limitations imposed by this type of investigation. Importantly, dissociated neuronal cultures form monolayers on MEAs thereby simplifying the modeling of network activity. Because of the small area of focus, morphological changes can be easily observed, stimulation can be precisely administered, and many survivability limitations of alternative preparations are eliminated allowing for long term testing.[1] Advantages of using electrical stimulation for the study of network activity include control of a closed system – no need for perfusion of chemicals, shorter recovery times, and ability to carry out a greater number of trials in a given period of time. Furthermore, prior research has indicated that correlated firing patterns in MEA could reveal network activity.[6,7] This thereby validates the idea that an *in vitro* preparation of PNS tissue could provide vital and realistic data on the inner workings of the human PNS.

OBJECTIVES

Our aim in this study is to provide a standard protocol for electrical stimulation that will replace chemical stimulation procedures previously used with the *i*CHIP. By adjusting the parameters of electrical stimulation, we sought to characterize the electrophysiological response of the PNS cultures and establish a convention for eliciting a reproducible spiking activity from the neurons. Furthermore, we sought to establish a relationship between spiking activity and variations in the stimulation parameters – amplitude, phase width, and phase shape. Of further interest is the relationship between conditions of the cell culture prep – days in vitro, media, and cell type – and the resulting activity.

MATERIALS & METHODS

General Procedure

1. Rat dorsal root ganglion cells and embryonic cortical neurons were harvested and cultured on MEA.
2. Cultures were imaged to determine location of cells relative to electrodes.
3. Stimulation and return electrode configurations were determined by cell location.
4. Stimulation parameters were adjusted within the ranges outlined in Table 1.
5. Each set of parameters was delivered in trains of approximately 10 pulses with 1 pulse every 2 seconds providing 10 “trials” per combination.
6. Viability assays were then conducted to correlate activity with culture health.
7. Data was then analyzed offline as described under Data Analysis.

Cell Cultures

For this experiment, we utilized two different culture preparations based on the cell types being tested. For the PNS cultures, adult rats were sacrificed and their spinal cords were removed and dissected. Dorsal root ganglion cells were carefully isolated and dissociated and finally plated at the base of the experimental well of the PNS device. For CNS cultures, embryonic cortical cells were purchased from a reputable vendor and cultured in the well for the PNS device.

Microelectrode Array

We utilized a MEA system developed and microfabricated at LLNL. Figure 1 shows the overall layout of the PNS component of the *i*CHIP. Figure 2 provides a picture of the actual device with a dime shown for scale (Figure 2 left). The MEA is magnified to show placement of each electrode (Figure 2 right).

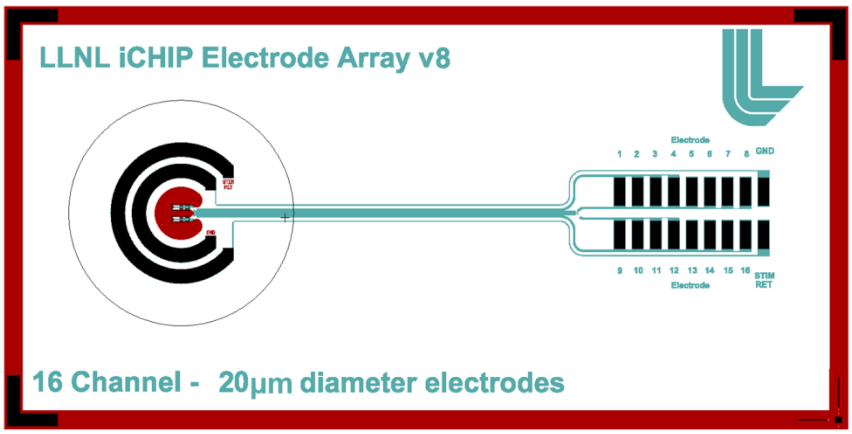

Figure 1: *Schematic depicting chip layout of LLNL* i*CHIP MEA for the PNS device. Gray circle illustrates placement of glass well over MEA. MEA consists of a global return electrode (outer black circle), reference electrode (inner black circle), and 16 thin-film electrodes insulated with polyimide (red). Compatible cables are hermetically connected to the connector (right) and interfaced with the electrophysiology data acquisition and stimulation system (Alpha Omega Multi-Channel workstation, not shown). AlphaLabSNR software was used to acquire and visualize data in real-time.*

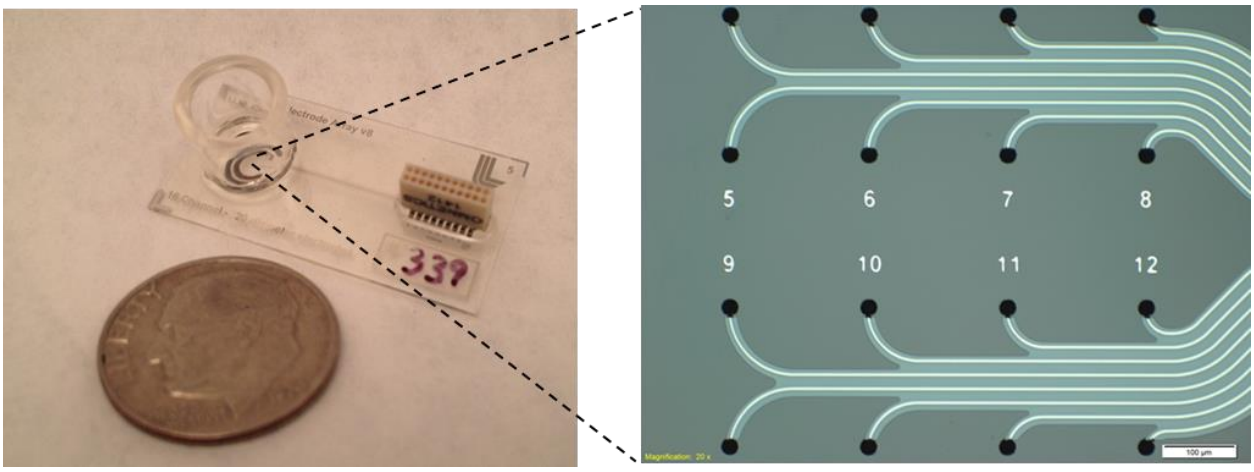

Figure 2: *PNS device shown with dime for scale (left). MEA at base of experimental well magnified and numbered to show position of electrodes 1-16 (right). Tissue samples are dissociated and cultured at the bottom of the well directly on top of the MEA. Electrodes are 20µm in diameter with 200-220µm separating adjacent electrodes allowing for precise stimulation and recording.*

Data Acquisition System

For out data acquisition system, Alpha Omega's Multichannel Workstation was utilized for obtaining continuous data sets of voltage changes over the course of the experiment. Voltages were recorded at a rate of between 22,000 and 44,000 samples per second. AlphaLabSNR software was used to visualize the data in real time, save the data sets, and set the parameters for stimulation.

Electrical Stimulation

Electrical stimulation was configured within the ranges outlined in Table 1 and delivered using a "push and hold" method. Each configuration of parameters was delivered in ten pulses with each pulse being delivered approximately two seconds apart.

Table 1			
Device Number	**516**	**533**	**568**
Cell Type	**rDRG**	**rDRG**	**eCNS**
DIV	**13**	**29**	**14**
Stimulation Parameters			
Amplitude (µA)	**1-1000**	**1-300**	**5-10**
Phase Width (µs)	**25-600**	**30-300**	**80-320**

Data Analysis

First, the raw electrophysiological recordings were visualized using NeuroExplorer (Figure 3). This allowed us to see the big picture and look for interesting activity.

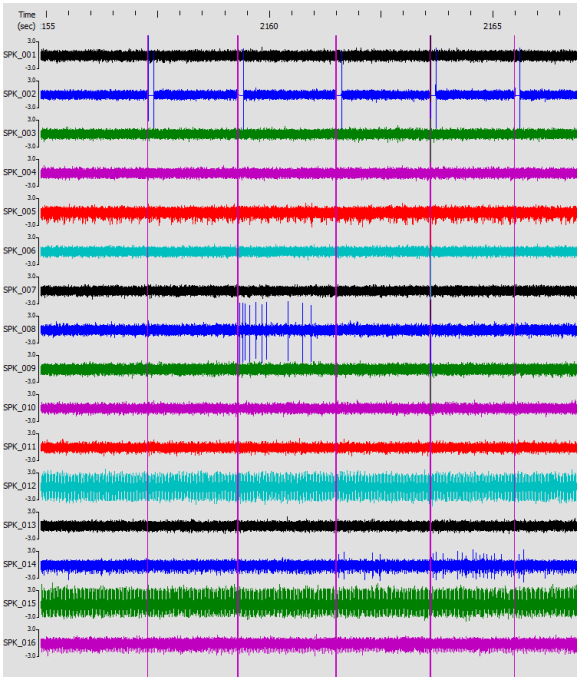

Figure 3: *Sample of raw electrophysiological recordings taken from one experimental run. Specifically, this data set came from the ten pulses delivered at 300 µA with a 300 µs phase width and a biphasic negative-positive pulse shape. Interesting spiking activity can be observed on channel 8 following the second pulse and channel 14 after the third, fourth, and fifth pulse.*

Second, pulse and spike waveforms were visualized and sorted using Plexon Offline sorter. Spike sorting provides a means of extracting activity from specific neurons out of the noise and clutter of activity. Different neuronal units will often have different waveforms. Plexon also provided us timestamps for all cross threshold events. The threshold could be adjusted to isolate the pulses and spikes (Figure 4 left, Figure 4 right, respectively).

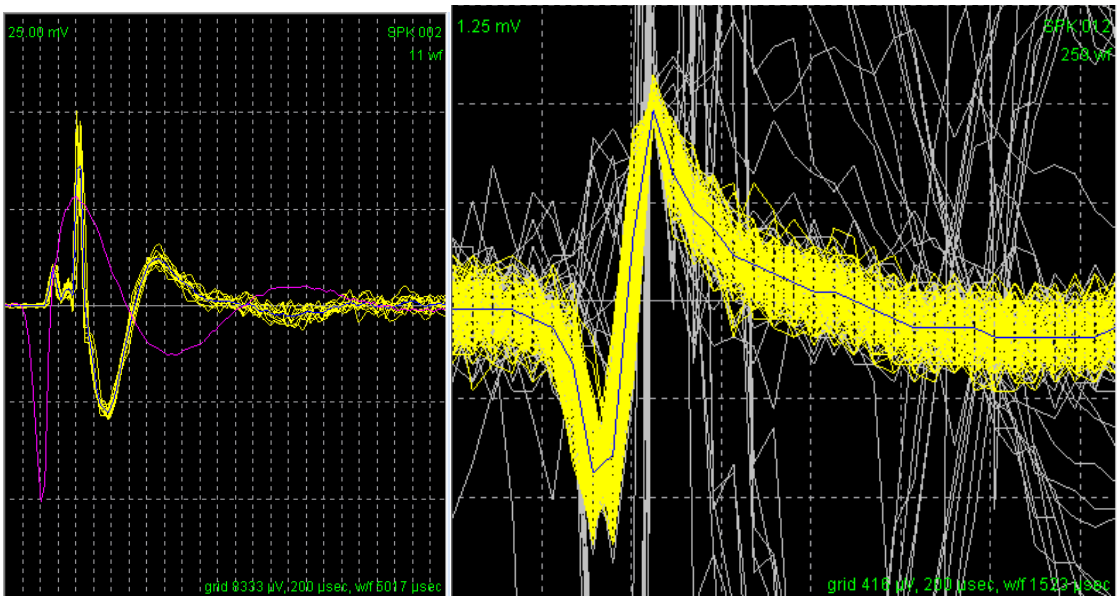

Figure 4: *Sample of waveforms depicted using Plexon Offline Sorter. Waveform of pulses (left) and spikes (right) shown to illustrate use of spike sorting for obtaining information about electrical activity with different shapes. This data sample comes from the experiment in which pulses were delivered at 300 µA with a 300 µs phase width and a biphasic negative-positive pulse shape.*

Pulse times and spike times were then plugged into MATLAB to calculate array-wide changes in firing rates following each pulse in a particular experimental run. MATLAB was also used to visualize those changes and interesting patterns were further investigated and analyzed (Figure 5).

Scripts written in MATLAB for analysis were validated by revisiting the NeuroExplorer data visualizations. This created a cycle in which data was viewed, analyzed, algorithms were tested, and revisualized.

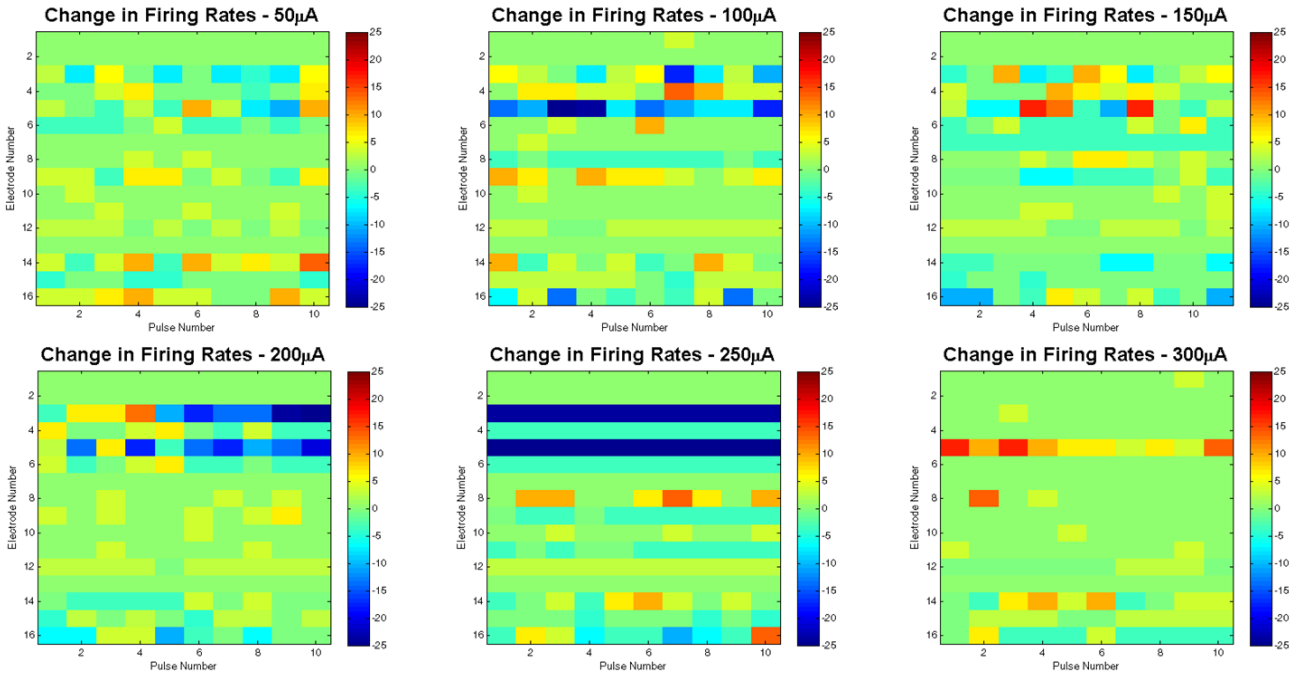

Figure 5: *Heatmaps depicting the changes in firing rates compared to a baseline recording following each pulse in an experimental run. The y-axis represents the electrode numbers (1-16 from top to bottom) and the x-axis represents the pulse number for a particular stimulation configuration. These heatmaps are examples taken from the varying amplitude experiments run on Device 533. Phase width was set at 300 µs and the shape was biphasic negative-positive. Amplitude was varied from 1 µA to 300 µA. Headers above each heatmap describe the run represented.*

RESULTS

Lethal Application of Stimulation

The first device tested, Device 516, was used to narrow our search for the second and third device tested. The range and number of parameters tested were highly varied. One of the key findings from that set of tests, however, was that 1 mA pulses applied directly on top of a neuron is lethal. This was found by comparing the level of spontaneous activity before

stimulation and after. The spontaneous activity was observed to immediately disappear following the first pulse at 1 mA. This observation was further corroborated by the results of the viability assay conducted post-stimulation. This finding therefore established an upper bound for stimulation in future studies which seek to avoid lethally damaging the cultured cells.

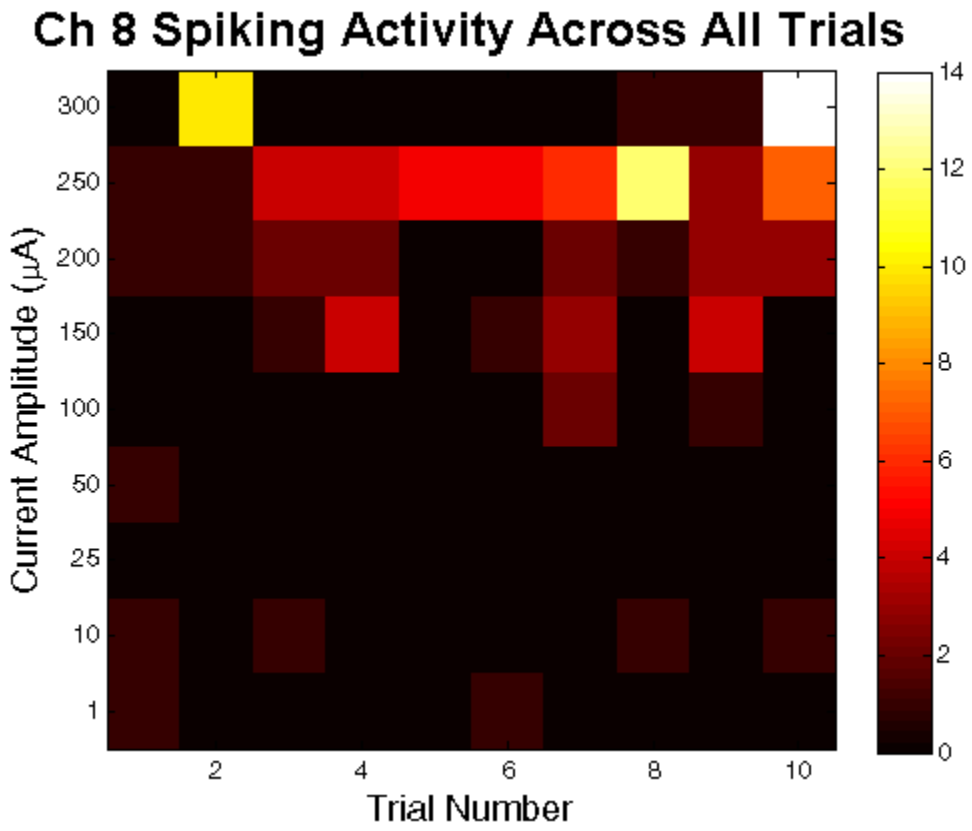

Figure 6: *Heatmap depicting the results from the experimental runs on Device 533 in which all parameters were held constant with only the amplitude of the current applied being changed. The y-axis represents the current amplitude being applied (1 μA at the bottom to 300 μA at the top). The x-axis represents the ten pulses delivered each experiment. Warmer colors are seen following pulses at higher amplitudes meaning that more spikes were*

Amplitude vs Max Spike Output

The maximum number of spikes observed following any individual pulse increased as the amplitude of the current delivered with each pulse increased. This can be seen in Figure 6 by the

gradual increase in the warmth of the colors observed moving up the heatmap. Figure 7 shows how the maximum number of spikes observed across all ten pulses for each experiment ran changed with increasing amplitude. The maximum number of spikes observed after any individual pulse was 14 which was seen following a 300 µA pulse.

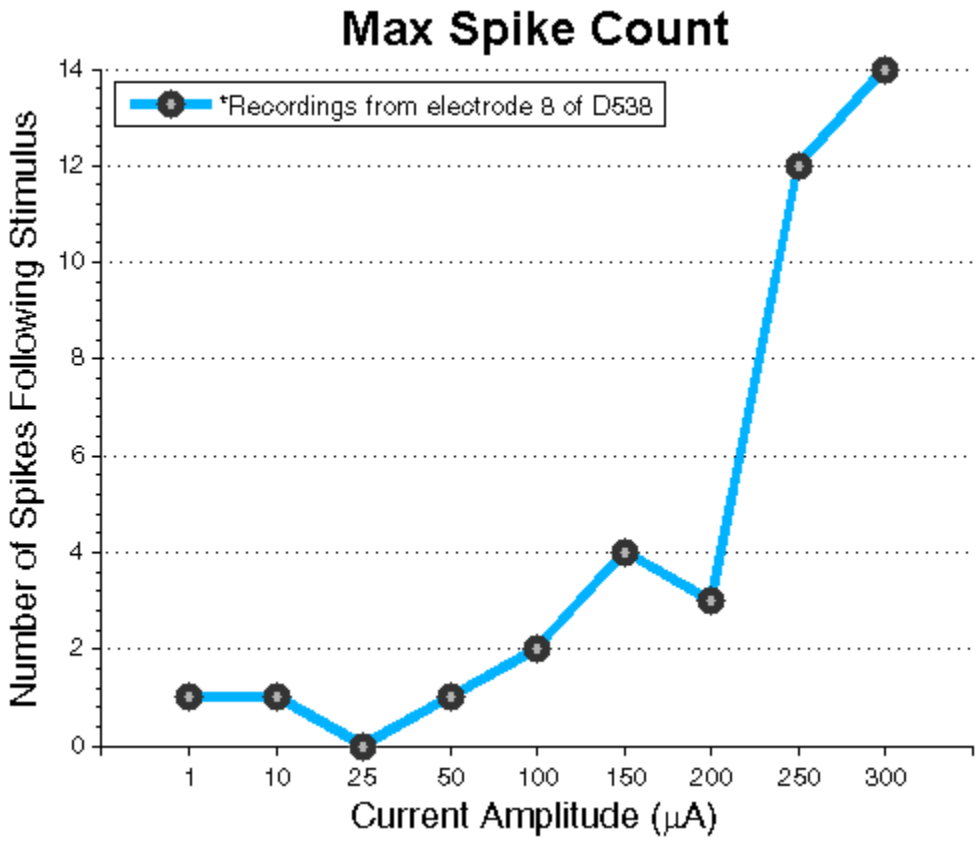

Figure 7: *Line graph depicting the maximum number of spikes observed following any individual pulse. The y-axis represents the number of spikes following the stimulus. The x-axis represents the current amplitude being applied. The maximum number of spikes counted after an individual pulse increased as the amplitude increased with 300 µA resulting in the maximum number of spikes.*

Best Amplitude for Consistent Spiking Activity

Interestingly, while the maximum number of spikes counted following any pulse increased with the current amplitude up to 300 µA, the average number of spikes counted peaked at 250 µA (Figure 8). This can also be seen on the heatmap from Figure 6. While the row for 300

µA has the warmest colors, most of the pulses were followed by zero to very few pulses, whereas, 250 µA pulses were consistently followed by some spiking activity.

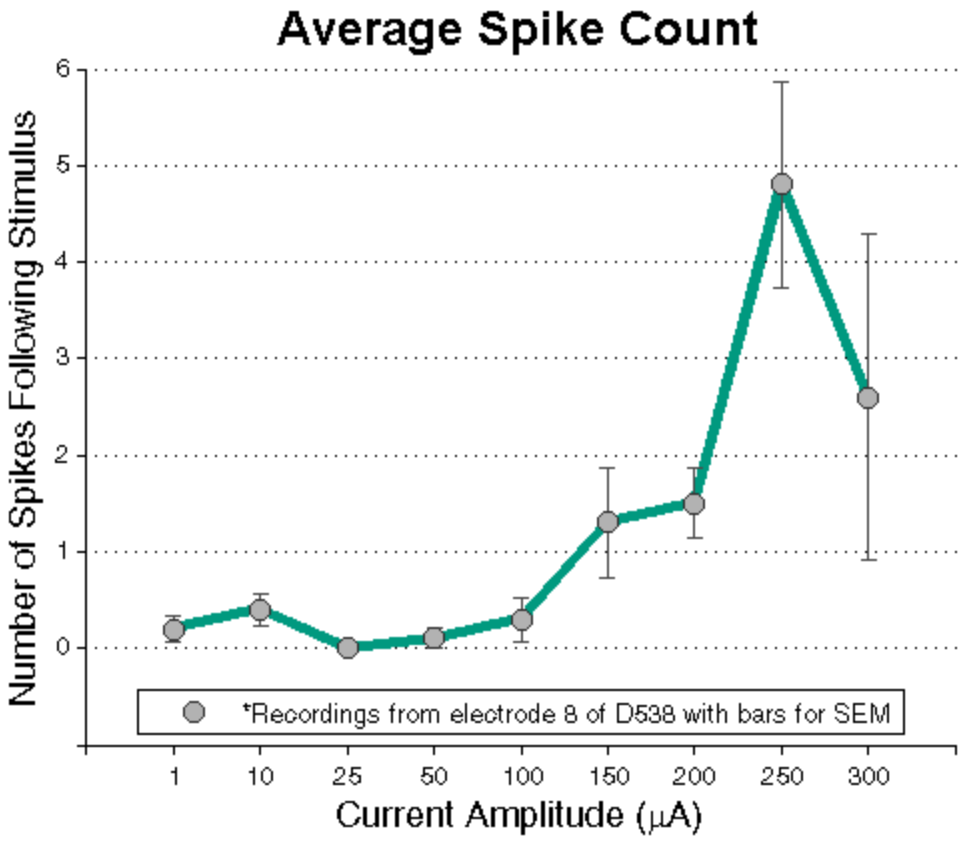

Figure 7: *Line graph depicting the average number of spikes observed following pulses at the specified amplitudes. The y-axis represents the average number of spikes following the stimulus. The x-axis represents the current amplitude being applied with the bars representing SEM. The average number of spikes counted increased with increasing amplitude of pulses delivered up to 250 µA. The average then dropped down at 300 µA.*

DISCUSSION

By varying the parameters for electrical stimulation, we were able to make several key observations. Increasing the current amplitude of pulses delivered to the culture increases the maximum spike count found following any individual pulse. Importantly, however, the average spiking activity appears to increase with the amplitude up to a point. We were able to get the most consistent activity following 250 µA. Also, we were able to establish an upper bound on

the level of stimulation we can reasonably apply without damaging the culture. 1 mA stimulation resulted in an instantaneous cessation of spontaneous activity observed in the culture indicating that 1 mA is lethal to the cells. We also found that younger cultures – fewer days in vitro – seem to be less excitable than more mature cultures and also show less spontaneous activity.

In future studies, it will be important to reproduce our findings to ensure the response curves we observed are reliable. We should also investigate the relationship between day in vitro and the excitability of the cells based on evoked spiking activity. We should also look at how frequency of stimulation affects the electrophysiological activity of the cultures. In line with the objective of the *i*CHIP project, we will also want to culture and stimulate human dorsal root ganglion cells.

SUMMARY OF PROJECT

Over the summer, we studied the effect of various electrical stimulation parameters on the resulting electrophysiological recordings of PNS and CNS cultures on the PNS component of the *i*CHIP system.

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