Undergraduate Research Thesis:

Novel Technology & Techniques to study Long-term Neural Dynamics of Living Neural Networks cultured in-vitro

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INTRODUCTION

Dissociated Cortical Networks Background (Advantages in using them as models)

A great deal of electrophysiology research aimed at understanding neuronal communication in single cells has yielded sufficient understanding of individual cells' role in communication. However, comparatively little is known about how *networks* of neurons interact with their environment to achieve function during development. The overarching objective of the work presented here aims towards quantifying the developmental changes synaptic connections *in-vitro* Living Neural Networks (LNNs) undergo under the influence of various controlled sensory environments.

The fundamental mechanisms of information processing and storage, and network plasticity for *in-vitro* LNNs are studied by monitoring extracellular electrochemical neuronal activity via a Multi-Electrode Array (MEA). MEAs monitor action potentials (defined as very rapid voltage transients across the neuronal membrane that turn sensory input into a discrete neuronal code), and enable observing morphological activities of *in-vitro* dissociated LNNs at the network level. Cortical neurons (extracted from 18-day old rat embryos) are dissociated and plated onto a grid of 59 electrodes on the center of an MEA culture dish. Once plated, the neurons reform synaptic connections with one another to become a single functional network, displaying highly correlated activity among constituent neurons. *In-vitro* LNNs can be maintained for up to two years [Potter et al. 2001], and therefore serve as excellent models for long-term experiments studying the development of network circuitry. The electrodes on an MEA are capable of both electrically stimulating neurons nearby, and recording extracellular, spatio-temporal action potential patterns from the LNN. Thus MEAs can be used as two-way interface between the outside world and LNNs.

The MEA is the primary electrophysiology tool for studying LNNs in this research endeavor. The primary advantage LNNs provide for studying neural network electrical and morphological dynamics stem from removing the neural circuit from a living organism to a reduced, artificial environment. This artificial environment enable scientists to study causal short-term and long-term developmental neural dynamics at the fundamental network scale. The field of electrophysiology aims to study the convoluted nature of neural networks by integrating concepts and techniques in the molecular biology, bio-chemistry, and bio-physics disciplines.

Studying long-term dynamics of LNNs in the *Potter Neuro-Engineering Lab* required us to develop novel technology and techniques previously capable of studying only short-term dynamics of LNNs. *This specific investigation focused on designing novel technologies aimed at studying a specific form of neural network plasticity: synaptic scaling in homeostatic plasticity* [Turrigiano et al. 2000], *within cultured in-vitro LNN.* Homeostatic plasticity is a cellular process that enables neurons to regulate their overall excitability to a set level (i.e. *how often* a neuron fires action potentials). Here we will use the MEA to stimulate neural networks in various *long-term* (hours to days) stimulatory environments and study the resultant developmental electrical (using electrophysiology technology) and morphological changes (microscopy technology). Electrophysiology techniques can be used to study and direct the electrical stability of these

LNNs. Synaptic scaling is a form of homeostatic plasticity and involves changes in quantity of AMPA receptors at the post-synapses of neurons that can be detected with two-photon microscopy techniques. Thus, developing a concurrent long-term electrophysiology & two-photon microscopy system enables us to study the correlated neural network electrical activity and morphological developments.

Significance and Applications

This research endeavor has broadened our understanding of the cellular mechanisms of information dynamics within LNNs. It also directly facilitated the progress of the parent NSF funded investigation's goal: to mimic the parallel real-time control capabilities of the brain by infusing neurobiological components into control systems (such as, but not limited to, a controller for supply/demand regulation of the electrical power grid). This investigation did this by broadening our understanding on how to restore LNNs dynamics to a state that is significantly more receptive to information content of training stimuli.

The results of this endeavor may have potential applications in medicine as well. Many neurological disorders are instigated when aberrant neuronal activity causes irreversible damage to the neuronal circuit at a critical developmental stage. Synchronized global bursting (explained in the Background section) occurs in mature neuronal circuits in the form of epilepsy [Wagenaar et al. 2005]; by broadening our understanding of the nature of synchronized bursting—and how to selectively inhibit adverse neuronal activity—we can further elaborate our knowledge of mechanisms resulting in epileptic form activity and provide avenues for its treatment.

BACKGROUND

Inhibiting Synchronous Bursting & its Benefits

Under non-stimulatory LNN training environments, *in-vitro* LNNs exhibit high rate of synchronous bursting (defined as an epoch when a network's constituent neurons increase their firing rate simultaneously) following synapse formation [Borodinsky et al. 2004]. Upon dissociation, the neurons plated onto the MEA lose the intrinsic architecture that defines the nature of synchronous activity for circuitry development—possibly causing the LNN to develop without architectural significance. Additionally, [Turrigiano et al. 2000] demonstrated that lack of activity in the form of synaptic communication mediated by action potentials within a LNN at crucial developmental stages can unnaturally strengthen synaptic connections by homeostatic plasticity mechanisms. These networks are driven toward an unstable state as homeostatic plasticity continuously up-regulates synaptic connection strength to compensate for a lack of external excitation. In stimulus-deprived cultures, this mechanism may cause network dynamics to become unstable, where any interaction between neurons often results in saturated synchronous bursts [Corner et al. 2006]. Indeed, our preliminary data indicate that stimulus-deprived LNNs often elicit similar, saturated bursting response to different stimuli—evidently disregarding the significance of information content within the stimulus. Attempts to train LNNs under these circumstances have proven difficult [Wagenaar et al. 2007]. This provides the motivation to alleviate stimulus deprivation in *in-vitro* LNNs and direct the development of network circuitry.

[Wagenaar et al 2005] demonstrated an inverse correlation between the frequency of stimulation and resulting synchronous burst rate during stimulation epochs. The objective of this research endeavor will attempt to scale this phenomenon to longer time scales and induce more pronounced and permanent network modifications. This form of long-term neural modulation manifests itself as a form of Homeostatic Plasticity known as Synaptic Scaling.

Global Synaptic Scaling

Homeostatic Plasticity is present in Neural Networks in two forms: 1) Pre-Synaptic Homeostatic Plasticity, where the network modulates its firing rates by altering the quantity of pre-synaptic neurotransmitter release from the pre-synapse and, 2) Post-Synaptic Homeostatic Plasticity, where the network modulates its firing rates by altering the number of post-synaptic neurotransmitter specific protein receptors.

Post-Synaptic Homeostatic Plasticity, also known as Synaptic Scaling, itself has two distinct mechanisms: Short-term local Synaptic Scaling, and long-term global Synaptic Scaling [Turrigiano et al. 2000]. This closed-loop process gives a neuron the ability to have global negative feedback control of synaptic strength of all its synaptic connections by altering the probability of glutamate (the most common excitatory neurotransmitter) making contact with post-synaptic AMPA receptors. Therefore a neurons' ability to modulate the quantity of post-synaptic AMPA receptors gives it the ability to achieve a set action potential firing rate.

The probability of glutamate making contact with a post-synaptic AMPA receptor is proportional to the concentration of both trans-membrane glutamate and post-synaptic AMPA receptors. When glutamate and post-synaptic AMPA receptors interact, the post-synaptic cell experiences a temporary depolarizing current, known as an EPSC (Excitatory Post Synaptic Current). Spatial and temporal accumulation of EPSCs at the post-synaptic neuron increases the likelihood of the neuron firing an action potential. Therefore the concentrations of extra-cellular glutamate (and other cations) and the quantity of post-synaptic AMPA receptors are directly correlated to a neurons' action potential firing rate. Some theories suggest each neuron uses calcium-dependent cellular sensors to detect their own action potential firing rate [Turrigiano et al. 2000]. These sensors also formulate input for cell-specific homeostatic plasticity regulation systems. In synaptic scaling, neurons use this information to determine a scale factor. Each neuron subsequently uses the scaling factor to globally scale (either up-regulate or down-regulate) the quantity of trans-membrane AMPA receptors at all post-synaptic sites.

A long-term, concurrent confocal microscopy and electrophysiology investigation conducted on cortical rat in-vitro neural networks (age > 3 weeks in-vitro) on in-vitro Multi Electrode Arrays showed the correlation between extra-cellular neural activity and AMPA receptor dynamics [Minerbi et al. 2009]. The long-term fluorescent microscopy tracked changes in quantity, density, and fluorescence of PSD-95 molecules over a time scale of 90 hours. Since PSD-95 molecules anchor to post-synaptic AMPA and NMDA receptors, they serve as reliable markers for post-synaptic transmembrane glutamate receptors. This investigation consisted of two sets of experiments, and monitored synapse-morphology and electrochemical behavior to spontaneous neural activity (i.e. no external stimuli or pharmaceutical manipulations perturbed the neural network). During this time the number of PSD-95 molecules increased almost linearly with time. The average fluorescence per molecule however remained constant throughout the 90 hours. The second experiment involved doing the same analysis, but with the addition of TTX (a pharmaceutical treatment used to block all post-synaptic action potential firing). During this time the quantity of PSD-95 molecules stayed constant, and the fluorescence of the PSD-95 molecules increased. This data indicates dissociated cortical networks (of at least 3 weeks of age in-vitro) modulate the concentration of AMPA receptors at post-synaptic sites to alter excitability rather than forming new synaptic connections.

Two-Photon microscopy Background

Two-photon microscopy is a fluorescence imaging technique that enables deep living tissue & long-term (hours-days). Two-photon microscopy uses near infra-red excitation light to excite fluorescent proteins transfected into cell cytoplasm prior to imaging. During each epoch of excitation, two photons of infra-red are absorbed. Two-photon microscopy techniques are used for long-term imaging to prevent photo-toxicity of living tissue.
Investigations aiming to study long-term dynamics of LNNs use similar electrophysiology and microscopy integrated experimental techniques as demonstrated in [Minerbi et al. 2009].

OBJECTIVE:

This research endeavor attempt to integrate electrophysiology and long-term microscopy techniques to quantify developmental changes associated with burst-inhibition and synaptic scaling. Additionally, new cell-culture environment control technologies aimed to facilitate long-term experiments were developed.

This research endeavor is broken into three thrusts:

1. **Thrust I: Cell-Incubation System:**
   Objective*: Enable Long-term electrophysiology & imaging experiments: Engineer & construct long-term environmental controller for keeping cells alive in custom-built environmental enclosure housing the two-photon microscope.*
2. **Thrust II: Electrophysiology:**
   Objective: *Conduct long-term (weeks) study to demonstrate whether it is possible to make a LNN burst less frequently (during non-stimulation epochs) if it developed at high stimulus frequency environment during early circuit developmental stages.*
3. **Thrust III: Enabling Two-Photon Microscopy Imaging—Transfection Protocols:**
   Objective: *Enable long-term AMPA receptor imaging for Two-Photon Microscopy: Develop LNN Transfection Protocol for Fluorescent Imaging.*

WORK PERFORMED

Thrust I: Cell-Incubation System

Objective*:*

Enable Long-term electrophysiology & imaging experiments: Engineer & construct long-term environmental controller for keeping cells alive in custom-built environmental enclosure housing the two-photon microscope.

Tangible Results:

1. Assisted in designing Environmental Controller
2. Constructed and maintained two Environmental Controllers

Summary:

The Hotbox is a custom-built environmental control device that modulates temperature, CO_2, and humidity levels inside a custom built environmental enclosure. Temperature is monitored with thermocouple, and a PID controller is used to drive heating elements. CO_2 monitored with CO_2 sensors; bang-bang controller is used to turn CO_2 air supply on/off.

Background

Two-photon fluorescence microscopy is a superior alternative to confocal microscopy (traditionally used in molecular biology imaging studies), as it enables researchers to *continuously* image cells for long periods of time (hours to days). This form of imaging is especially useful for studying neural circuit developments over long time periods. Long-term two-photon microscopy requires stable long-term atmospheric control, specifically O_2, CO_2, humidity, and temperature levels. [1] sufficiently slows cell-culture media evaporation and osmolality change rates for short-term two-photon microscope recordings. Continuous control of temperature and CO_2 (with the MEA lid) enables long-term imaging of these LNNs on MEAs.

Conventional incubator geometries are not fully equipped to house fully functional two-photon microscopes. Additionally, since many two-photon microscopes are custom built, it is very difficult for incubator manufacturers to address this predicament. It is also very expensive to custom order an incubator. Here we provide an inexpensive [~$2000] and more flexible home-made alternative for long-term CO_2 and temperature control. Figure 1 displays our home-made Hotbox and a custom built environmental enclosure (also home-made) enclosing our two-photon microscope. Although we have tailored the functional needs of this system specifically to two-photon microscopy and calcium dye microscopy users, it can be used for any cell-culturing environment control demand. Temperature levels inside the environmental chamber are monitored with a thermocouple, and a PID controller is used to drive heating elements to turn the heat on or off. CO_2 level inside the environmental chamber are monitored with CO_2 sensor; a bang-bang controller reads this temperature input and controls a CO_2 gas valve to regulate

CO_2 air flow.

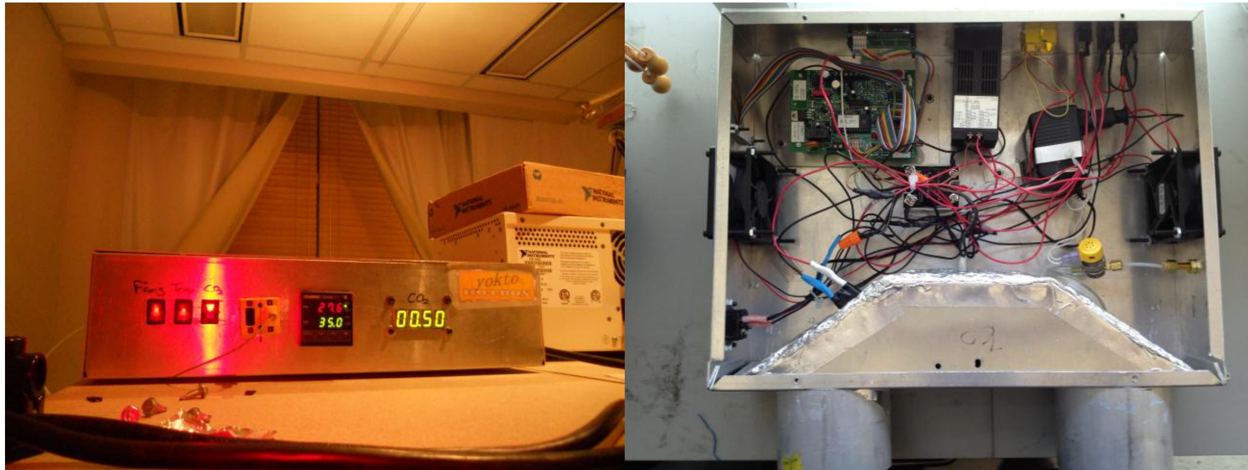

Figure 1a: Environmental Controller. Left: Front view. Right: Top view showing layout of devices comprising the environmental controller.

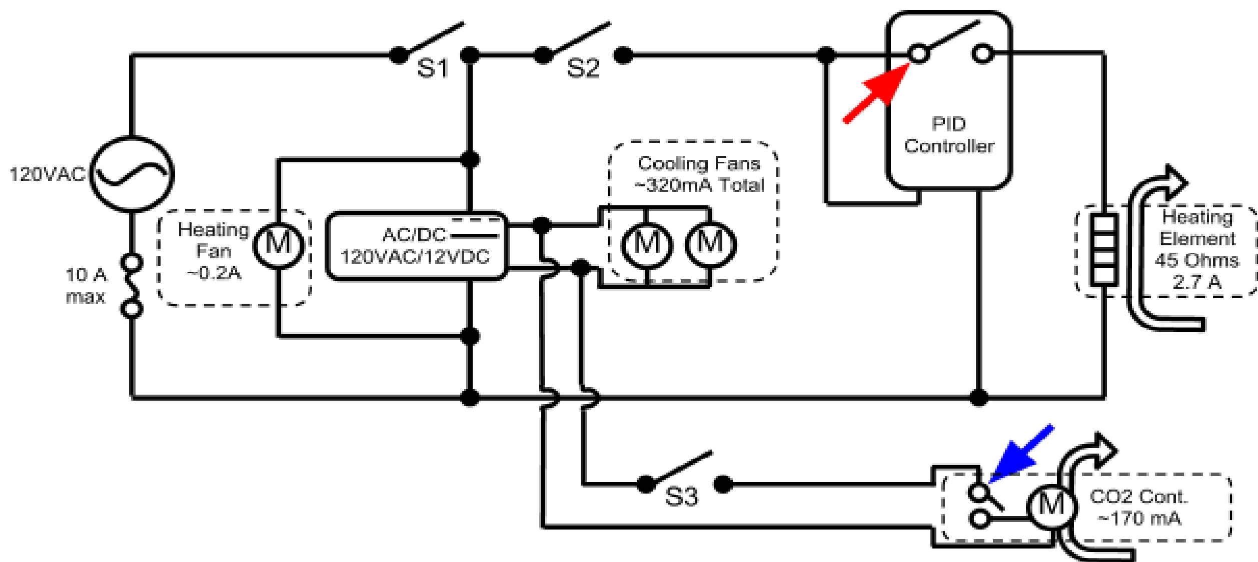

Figure 1b: Environmental Controller Circuit.

Defining Environmental Controller Functions:

1) Environment (Temperature & CO2 control): The environmental controller must maintain physiological environment for living mammalian cells. The two most important physiological components for regulating cell health are Temperature and CO_2 control. [1] Sufficiently slows cell-culture media evaporation and osmolarity change rates for short-term two-photon microscope recordings. We find continuous control of temperature and CO_2 (with the MEA lid) enables long-term imaging of these DLNNs on MEAs for our experimental setups.

2) Geometrically Flexible: The spatial design of the enclosure should accommodate to systems of any shape and size in order for it to be implementable for our three systems. Conventional incubator geometries are not fully equipped to house fully functional two-photon microscopes due to volume constraints.

3) Prevent thermal drifting: Our two-photon microscope stage contains materials (glass lens, metals, etc) with different thermal masses. Sporadic changes in temperature can cause these different materials to expand at different rates—and cause permanent damage to expensive equipment. This means we can't have the temperature levels change too sporadically.

4) No experiment interference: The environmental unit must not inhibit our ability to do microscopy (specifically light sensitive microscopy, one and two photon fluorescent light microscopy, or other light microscopy), electrophysiology, or pharmacology experiments.

5) Upright & Inverted microscope friendly: The environmental unit must be implementable to both up-right and inverted microscope setups. Our two-photon microscope is an up-right microscope.

6) Cost Efficient: Cost less than $2000.0 to make with readily available parts.

Although most of the currently available conventional cell-culture incubators provide the *some* of the aforementioned environmental qualities, the few that do meet the functional criteria fail meet the Cost Efficiency criterion, and are inflexible in terms of geometrical modifications to accommodate a wide range of microscopes. See Table 1 for a function-means table for some popular available models and our hotbox.

Function → Product	Temperature & CO_2 control	Geometrically Flexible	Prevent thermal drifting	Electrophy/ Imaging/ patch	Upright & inverted friendly	Perfusion
Hotbox	Yes	Yes	Inconclusive	Yes/Yes/Yes	Yes	No
Automate Scientific Brain Chamber*	Yes	No	N/A	No/Maybe/no	No	Yes

Table 1: Functions chart by vendors

Bioptech, Delta T Controlled Culture Dish System	No CO_2	Yes/but it is difficult	N/A	No/ Yes/ Maybe	Yes	Yes
Campden Inst Model 7800	No CO_2	Yes	N/A	Yes/Yes/Yes	Yes	Yes
Campden Inst Model 745	No CO_2	Yes	N/A	No/Yes/Yes	No	yes
Warner Inst DH-35/40	Yes (CO_2 perfused)	Yes	N/A	Yes/Yes/Yes	Yes	Yes
Warner Inst HMW	Yes (CO_2 perfused)	Yes	N/A	Yes/Yes/Yes	Yes	yes

Materials & Construction

Enclosure:

Bill of Materials for Enclosure

1. Reflectix insulation
2. Wooden beams (5/8" X 5/8" X 3')
3. Angle brackets
4. Adhesive-backed Velco strips (3/4" wide)
5. Adhesive backed foam insulating strips (1/2" wide)
6. Dryer ducting (3" diameter)

A custom enclosure can be constructed by making a sturdy skeleton with wooden beams. The skeleton should be dimensioned to take movements of the microscope into account. During the frame designing and construction process, we used hinges to make a door to allow accessibility inside the enclosure.

After construction of the skeleton, we used two layers of reflectix insulation to insulate four walls and the ceiling of the enclosure. We then placed the enclosure on a floating-nitrogen table stationing our microscopy equipment. We then used one layer of reflectix insulation to insulate the floor of the enclosure. Next we used adhesive backed foam insulating strips on the door-frame to create an air-tight seal on the enclosure to ensure the enclosure is airtight when the door is closed. Additionally, we cut out some reflectix insulation to make windows. Magnetic strips were attached around the perimeter of the window to interface with the reflectix window cover (with matching shape of magnetic strips. This gave us access to our equipment without having to open our doors—this is especially recommended since our experiments required a great deal of preparations and modifications at the stage area with minimal mechanical

movement during experiments.

Once the skeleton was insulated, we cut two holes in through the wall of reflectix insulation to enable air circulation to and from the enclosure. The first circular hole was cut at any back wall corner. Subsequently, cut the second hole at a back wall corner such that it is diagonal to the first hole. Connect the bottom hole to the hose providing air to the enclosure. Connect the top hole to the hose sending air back to the hotbox.

Environmental Controller

See Figure 1b for the hotbox circuitry, and Figure 1a to see the physical layout inside the hotbox. The hotbox maintains CO_2 and temperature levels inside an enclosure. The hotbox maintains a continuous air flow (the rate of air flow depends on the volumetric air flow of the heating fans). The heating fans inside the heating-chamber of the hotbox create a directional air current. "New" air exits the heating-chamber of the hotbox and enters the enclosure at a bottom corner hose, while the "old" air exits from the enclosure via the top corner hose.

Bill of Materials for Hotbox:

1. Power-socket with fuse-box: Provides power to the hotbox. The fuse-box provides safety measure.
2. Cooling Fan switch: This switch enables turning AC powered cooling fans (#10) on/off
3. Temperature system switch: This switch allows passage of current to the temperature PID controller (#5), heating elements (#17), and the external solid state relay (#8).
4. CO_2 system switch: this switch allows passage of current to the CO_2 bang-bang controller (#12).
5. PID Controller (Model CNi-1644-C24 from Omega Engineering): A PID controller (Proportional-Integral-Derivative) is a control system that calculates the difference between the temperature set-point and actual measured temperature. This device receives input from a thermocouple, uses a built-in cold-junction to determine temperature value. The PID controller then sends a pulse-width modulated current to drive an external solid state relay (SSR), which drives the heating-elements. The PID controller analyzes and minimizes this error over time.
6. Type-K thermocouple (SA1XL Series 5-Pack**)**: A thermocouple detects changes in temperature by monitoring the proportional changes in voltage between two different metals (usually Chromium and Aluminum). The adhesive material on the thermocouple is attached to the stage near the cultured cells. The other end is connected to the PID controller.
7. Thermocouple panel mount: To connect the thermocouple to the PID controller
8. External Solid State Relay (SSRL240 and SSRL660 Series from Omega Engineering): Receives input from PID controller, and drives heating elements.
9. AC/DC Converter: Used to provide DC power to CO_2 sensor, and two cooling fans.

10. Cooling fans (AC, plastic): Used to cool temperature in the main circuit area inside the hotbox.
11. CO_2 Sensor & CO_2 Bang-bang controller (Model 300 CO_2 Sensor from Digital Control Systems): Receives CO_2 level input from CO_2 sensor #11, and employs a bang-bang control algorithm to drive a CO_2 gas valve, #14.
12. CO_2 level display: Displays detected CO_2 level
13. Clippard gas valve: Receives input from CO_2 bang-bang controller (#12) on whether to allow CO_2 glass flow go to enclosure.
14. Heat-chamber walls: Sheet metal is reshaped and riveted to form a trapezoid shaped heating chamber. The heating chamber will then be thermally insulated with reflectix material.
15. Braided-fiberglass wire insulation (Not shown)
16. Nichrome-wire heating elements: The means of regulating temperature.
17. Heating fan (metal): To provide air-flow
18. Flanges
19. Thermal fuse: Added safety measure; shuts off all power supply if temperature of air rises past a threshold.
20. Project box.
21. Brass Fittings: Temperature proof.

Temperature Control and Results:

Temperature is monitored with thermocouple, and a PID controller is used to drive heating elements to turn the heat on or off. A type K thermocouple (from Omega Eng.) measures the instantaneous temperature value at its location, and sends temperature input value to a PID controller (from Omega Eng.) with a built-in cold junction. The PID controller then drives an external Solid State Relay (from Omega Eng.) to drive a coiled Nickel-Chromium wire heating element. The external SSR send a pulse-width modulated current to turn the heating elements on/off. A heating element yields 800 Watts of heating power.

Figures 2a,b,c displays the temperature dynamics of an environmental chamber with our two-photon microscope driving and controlling heat flow. Figure 2a displays the ramp up time (~6000 seconds) reflects the amount of time it takes for the Environmental Controller to reach its temperature set-point (35°C) with the air and contents within the Environmental Chamber initially at room temperature. Once this set-point is reached, the PID controller then maintains the temperature at ~35°C. The specific ramp up times and oscillation periods of the temperature is dependent on the specific PID tunings, thermal mass of the contents inside the Environmental enclosure, the volume of the Environmental Enclosure, the power of the Heating Elements are driven at, and quality of insulation of the Environmental Enclosure. Proper PID controller tuning can empirically compensate for the latter sources of temperature variations.

Figure 2b displays two periods of temperature fluctuations when the front panel of the Environmental Chamber is kept open for short periods of time. Figure 2c displays the one period

of temperature fluctuations when the front panel of the Environmental Chamber is kept open indefinitely.

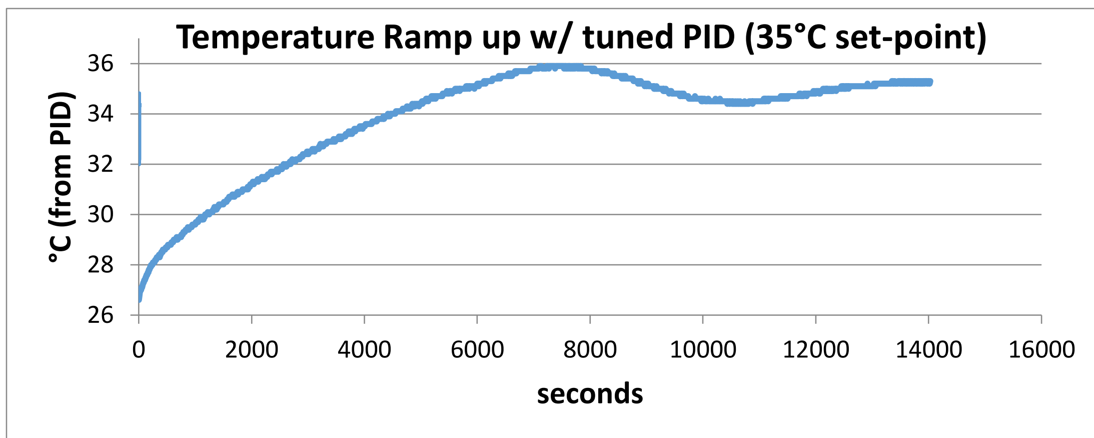

Figure 2a: Temperature Ramp up and oscillation with a tuned PID controller with 35°C set-point.

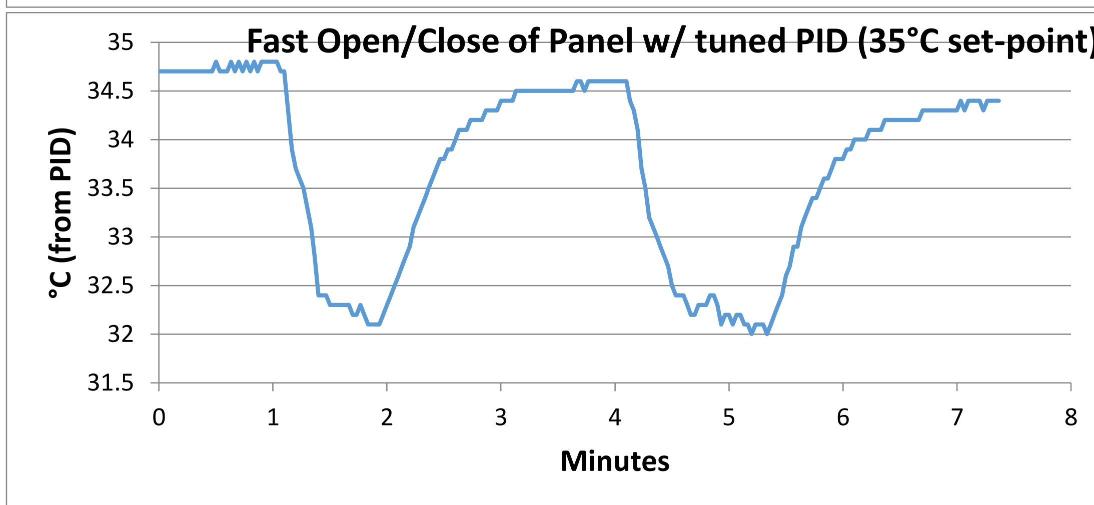

Figure 2b: Temperature oscillations when front panel is opened and closed within a ten second window with a tuned PID controller with 35°C set-point.

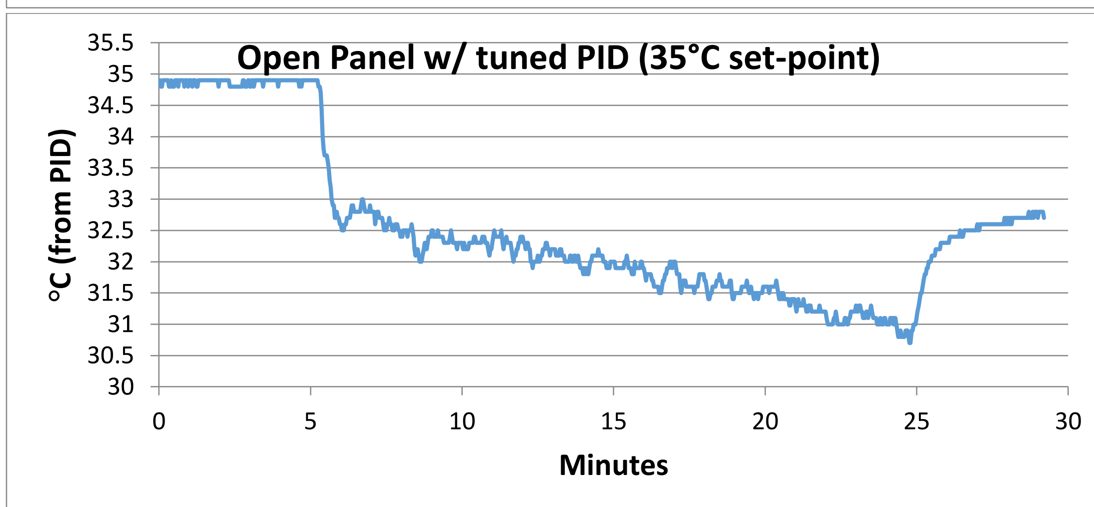

Figure 2c: Temperature oscillations when front panel is left open with a tuned PID controller with 35°C set-point.

CO2 Control and Results:

The CO_2 control system consists of a CO_2 Sensor (Digital control systems; Model 300), CO_2 Controller (Digital control systems; Model 300), and a CO_2 gas valve (Clippard Gas valve). The CO_2 bang-bang monitoring system receives instantaneous CO_2 level input from a CO_2 sensor. Based on the set point, if actual CO_2 values are above or below a set threshold level, then the bang-bang controller sends a current to turn the gas valve on or off. The CO_2 valve must be connected to a CO_2 gas regulator (with 10-20 psi continuously) and to the heating-chamber.

Table 2 displays results from CO_2 duty cycles and CO_2 overshoot values at varying gas release pressures and open/closed panel conditions.

CO_2 Valve Duty Cycle (Table 2)	
Closed Panel & Door at 20psi	
Duty Cycle (avg of 3 cycles):	11.4%
Duty Cycle Period:	320 seconds
Max/Min CO_2 levels:	6.3% / 4.9%
closed panel & door @ 12psi	
Duty Cycle (avg of 3 cycles):	18%
Duty Cycle Period:	260 seconds
Max/Min CO_2 levels:	5.8% / 4.8%
open panel & closed door @ 20 psi	
Duty Cycle (avg of 3 cycles):	90%
Duty Cycle Period:	265 seconds
Max/Min CO_2 levels:	5.1% / 4.0%

Conclusions:

The constructed environmental controller has been operating for two years with minimum-moderate maintenance effort. Post-construction maintenance often addressed safety concern issues that originated from the heating elements; there have been incidents when the air temperatures reach dangerously high levels (past the set-point). These failures originated from air-circulation fan, PID controller, and heating elements malfunctions. It is important to ensure the quality of these components, as any operational failure can induce dangerous health risks and damage to the system (and those surrounding it). Air-Circulation fan malfunction stop driving air currents, causing the heated air to stay localized within the hotbox—thus overheating the electronics. Improper PID tunings can not only reduce the effectiveness of environmental control of this system, but it can also drive the heating elements to dangerously high temperatures. In addition to ensuring all components can operate at incubated temperatures, a thermal fuse (rated at 120°C) was added in series to the systems power

supply. This fuse is placed near heating elements, and breaks the circuit when local temperatures exceed 120°C.

Thrust II: Electrophysiology

Goal:

Conduct long-term (weeks) study to demonstrate whether it is possible to make a LNN burst less frequently (during non-stimulation epochs) if it develops in a high stimulus frequency environment during early circuit developmental stages.

Tangible Results:

1. Formulate novel experimental protocols to empirically demonstrate mechanisms of the Synaptic Scaling phenomenon.
2. Successfully conducted preliminary experiments demonstrating long-term high frequency stimulation treatments alleviating bursting behavior.

Summary:

Attempts to scale short-term Burst-Inhibiting phenomena to longer time scales and induce more pronounced and permanent network modifications were successful. However, the integrity of LNN health was compromised at 17 DIV; thus this experiment can only serve as a preliminary experiment for future experiments attempting to demonstrate Burst-Inhibiting and Synaptic Scaling phenomena.

Long-term High-Frequency Stimulation to Inhibit Bursting

As mentioned in introduction section, [5] demonstrated lowering bursting activity within LNNs by administered stimulation pulses at 10Hz. These results are limited to demonstrating this burst alleviating effect only during epochs the LNN was stimulated in.

One of the main goals of this investigation was to demonstrate whether it is possible to make a LNN burst less frequently (during non-stimulation epochs) if it developed in a high stimulus frequency environment during early circuit developmental stages. An experiment successfully demonstrated the induction of long-lasting alterations in neuronal activity by stimulating a LNN with spatially distributed, square-wave voltage pulses at random times with an average rate of 10Hz (square-wave pulses have been shown to maximize the action potential response [6]) throughout its development, when compared to a LNN that received no stimulation at any point of its development. The LNN receiving the burst inhibiting treatment was stimulated for 23.5 hours/day, and received no stimulation for 0.5 hour/day (due to media changes).

Figure 3a displays the ASDR (Array-wide Spike Detection Rate—defined as the sum of number of action potentials detected on all the electrodes on the MEA *per second)* for *spontaneous activity* of two LNNs over a 30 minute epoch. The first LNN (red line) received random burst inhibiting stimuli throughout its development; the second LNN received no stimuli (blue line; extracted from [7]; conducted in near identical conditions). Both recordings were

taken during periods when no stimulus (30 minute epochs) was delivered; therefore the qualitative change in the nature of activity patterns observed in the LNN that received stimulatory input during development is a result of changes to the network itself, and not to presence of a burst inhibiting stimulus during recording.

Figure 3b displays the overall firing rate (spikes detected per second) throughout the development (17 days) for the LNN receiving burst inhibiting stimulus versus the LNN receiving no stimulation. The overall low level of median ASDR indicates the burst inhibiting stimulus having its intended effect. This claim can be validated with a Burstiness Index (BI) analysis (Figure 3c). The Burstiness Index is defined as the fraction of all action-potentials manifesting themselves in a synchronized burst form. The BI indicates that the burst-inhibiting stimulus took on its intended effect gradually throughout the LNNs life-span.

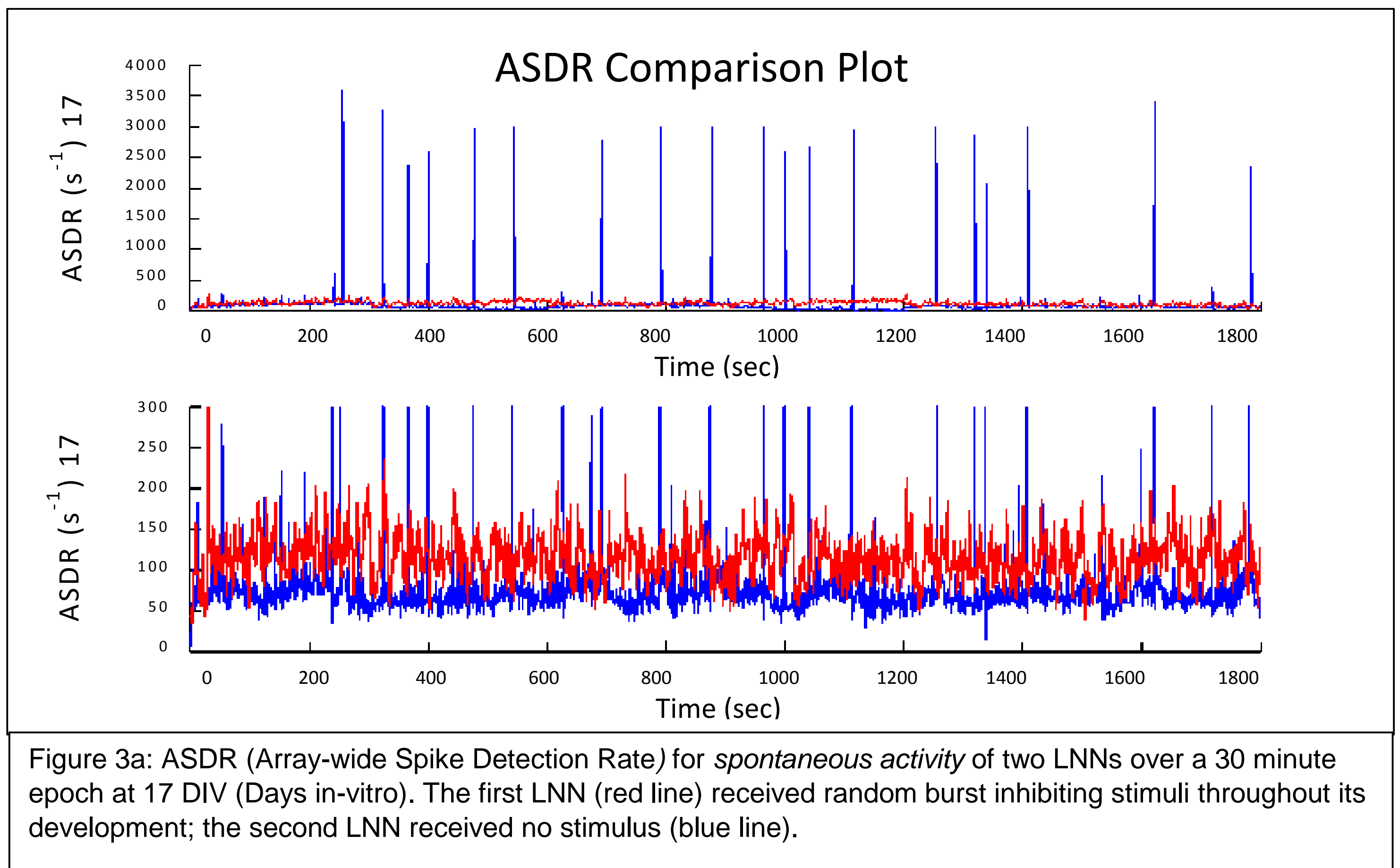

Figure 3a: ASDR (Array-wide Spike Detection Rate*)* for *spontaneous activity* of two LNNs over a 30 minute epoch at 17 DIV (Days in-vitro). The first LNN (red line) received random burst inhibiting stimuli throughout its development; the second LNN received no stimulus (blue line).

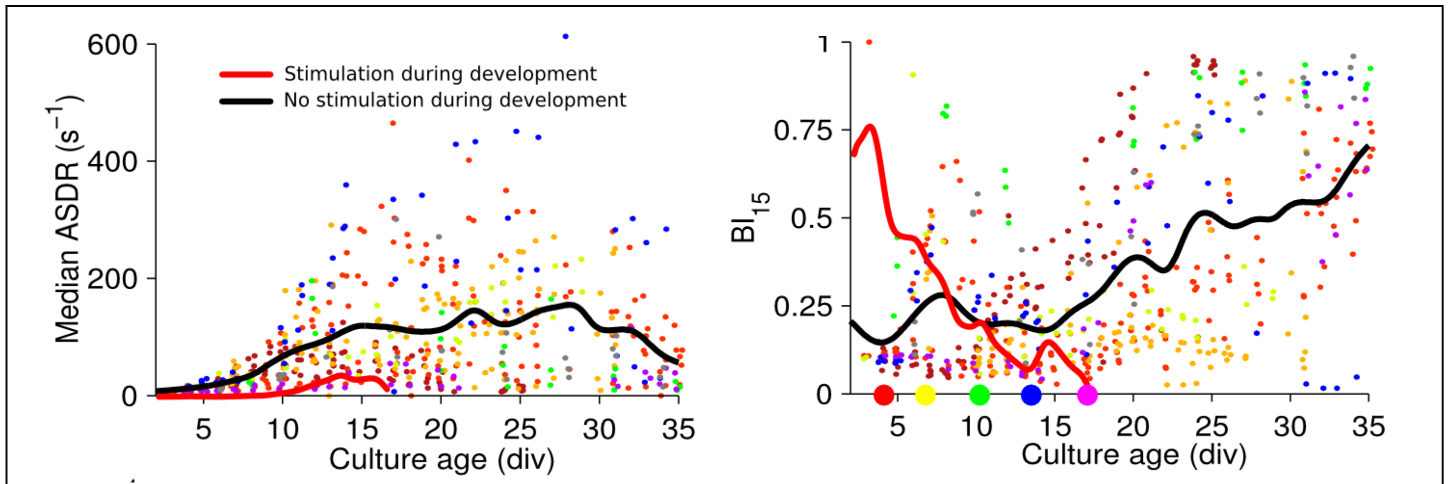

Figure 3b: (Left) Long-term ASDR (Array-wide Spike Detection Rate*)* of two LNNs over a 35 day epoch. The first LNN (red line) received random burst inhibiting stimuli throughout its development; the second LNN received no stimulus (black line).

Figure 3c: (Right) Burstiness Index comparison of two LNNs over a 35 day epoch. The first LNN (red line) received random burst inhibiting stimuli throughout its development; the second LNN received no stimulus (black line).

Conclusions

Although the two LNNs were derived from different rat embryos, it still qualitatively demonstrates that bursting can be alleviated when they are cultured in high frequency stimulation environments during early circuit developmental stages. Further quantitative analysis was not performed for the same reason.

This experiment can serve only as a preliminary experiment for two primary reasons: 1) the LNNs were cultured from two different rat embryos; therefore for analysis quality purposes, these comparisons must be reproduced for sister cultures (LNNs derived from the same rat embryo). 2) The health of the LNN receiving burst-inhibiting stimulation treatment was compromised at day 18. Qualitative microscopy images verify LNN health started debilitating on day 18. Thus, the extent of these results are only exist from days 0 to 17. Future comparisons should consider differences in activity dynamics at more mature stages of circuit development.

This preliminary experiment validated our hypothesis: it is possible to induce long-term circuitry changes affect spontaneous burst inhibiting behavior (up to 30 minutes) using high frequency stimulation treatments. This demonstration opens many avenues enabling future investigators to induce an “observing state” within the LNNs via the use high-frequency stimulations as means of communicating and training LNNs.

Thrust III: Enabling Two-Photon Microscopy Imaging—Transfection Protocols

Goal:
Enable long-term AMPA receptor imaging for Two-Photon Microscopy: Develop LNN Transfection Protocol for Fluorescent Imaging.

Tangible Results:
1. Developed plasmid electroporation technique for LNNs to transfect plasmid vectors into dissociated-rat-cortical-neurons.
2. Imaged fluorescent tagged neurons in-vitro using Confocal Microscopy.

Summary:
Electroporation based transfection techniques were assessed for their effectiveness for fluorescent microscopy imaging cases. Although some electroporation parameters yielded successful transfections, all treatments yielded low transfection efficiency and low cell viability.

DNA-Vector Plasmid Transfection Techniques & Results:
In order to study the Synaptic Scaling phenomenon using fluorescent microscopy, changes in the quantity of post-synaptic AMPA receptors must be detected in time-lapsed style.

Traditional cytoplasmic fluorescent proteins cannot enable detecting changes in quantity of post-synaptic AMPA receptors.

In order to perform fluorescence microscopy on living neurons, plasmid DNA vectors containing fluorescent proteins must be transfected (the process of inserting plasmid DNA vectors into a cell) into the cell cytoplasm. The objective was to image neurons with a fluorescent protein called gluR2-SEP. GluR2 is a subunit of the AMPA receptor proteins. By tagging this part of the protein, investigators can effectively assess the quantity and locations of post-synaptic AMPA receptors. Due to the size of the GluR-2 proteins, detection of this fluorescent protein on a fluorescent microscope is difficult without appropriate physical filters enabling imaging at high signal-to-noise ratios; and during this time period of the research endeavor, our two-photon microscope could detect cell fluorescence with only very low signal-to-noise ratio. All attempts to assess the effectiveness of transfection and imaging techniques for imaging post-synaptic AMPA receptors using two-photon microscopy were inconclusive.

As a proof of concept, transfection and imaging technique effectiveness were assessed using a Confocal Microscope. The table below (Table 3) displays a qualitative assessment of the transfection efficiencies under different electroporation protocols using a confocal microscope. Even with the high signal-noise ratio capability of the Confocal Microscope, it was difficult to determine if transfection protocols were successful or not for the GluR2-SEP transfected groups (experiments 1 and 2). Therefore, experiments 3 and 4 aimed at studying transfection efficiencies with Ds-Red cytoplasmic fluorescent proteins.

The last row of Table 3, yielded positive results at 150V electroporation voltage. Confocal microscopy images of the neurons transfected with Ds-Red are shown in Figures 4a and 4b.

Additionally qualitative assessments of cell viability were made by comparing cell density of electroporated cells versus non-electroporated cells. Cell density on plated MEAs for all electroporated LNNs were orders of magnitude less than in non-electroporated LNNs.

Electroporation Parameters (Table 3)								
Electric Pulse Generator	Cell-culture Density	Voltage (V)	Time (ms)	Capacitance (Farads)	Plasmids Used	Plasmid Concentration	Transfection Efficiency	Cell Viability
Decaying Pulse	500 cells/uL	250,500	-	50uF	GluR2-SEP	12ug	0%	Very Low
Square Pulse	500 cells/uL	250	18	-	GluR2-SEP	3ug	0%	Very Low
Square Pulse	2500 cells/uL	250,300	18	-	Ds-Red	3ug	0%	Very Low
Square Pulse	2500 cells/uL	**150**, 250, 300	18	-	Ds-Red	3ug	5-10%	Low

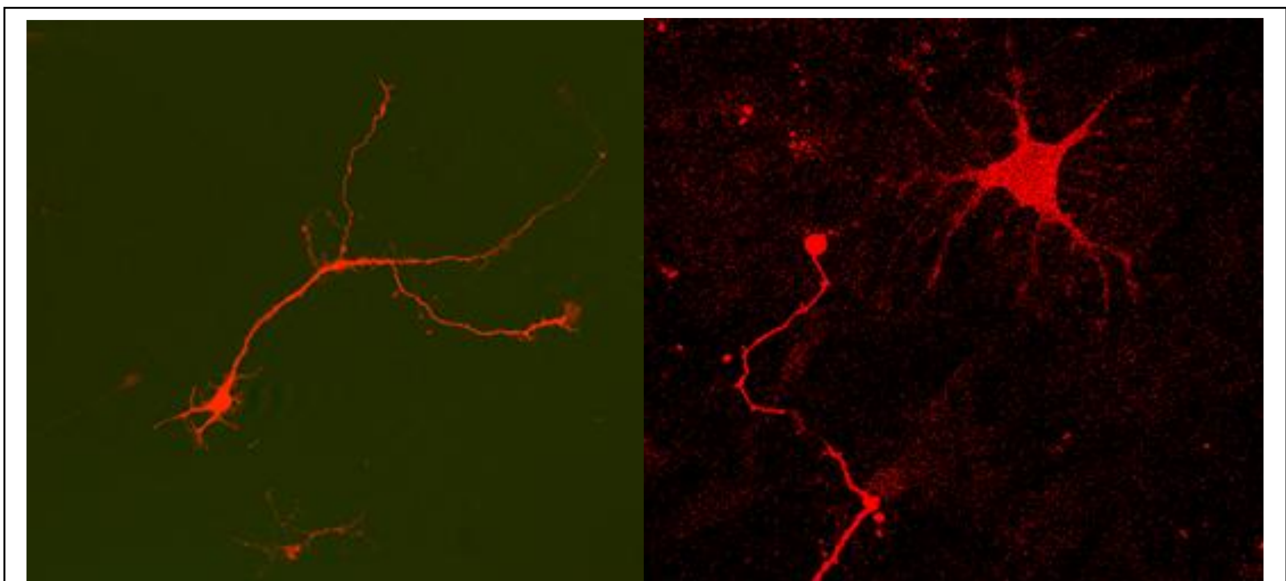

Figure 4a: (Left) Confocal microscopy imaging for Ds-Red fluorescently labeled neuron in a LNN.

Figure 4b: (Right) Confocal microscopy imaging for Ds-Red fluorescently labeled neuron and glial cell in a LNN.

Conclusions

Although neurons in LNN were successfully transfected via electroporation techniques, it is not a recommended form of transfection for studying computational dynamics of LNNs. In addition to the sensitive transfection efficiency responses at various electroporation parameters, all experiments yielded very low viability. It is a well-known fact that cell culture health is harshly compromised from high voltage parameters in electroporation techniques. This experiment demonstrates this severity of damage makes electroporation a poor choice of transfection for LNNs.

A possible source for severe cell death during electroporation can be due to the presence of extra-cellular media during the electroporation treatment. Lonza Biosystem's state 50-70% cell viability yields following electroporation. A difference in the method is the use of a specialized electroporation media (designed to mimic the intra-cellular liquid composition of the desired cell type). Electroporating cells surrounded by extracellular media causes intra-cellular and extra-cellular chemical concentrations to combine—altering the delicate chemical trafficking going in and out of the cell—thus damaging cell health. Future investigations attempting to use electroporation techniques to transfect neurons should conduct investigations on using appropriate intra-cellular media.

Conclusions

The overarching goal of this endeavor was to develop novel technologies and techniques to enable studying Synaptic Scaling in LNNs. Although, this endeavor harbored electrophysiology and two-photon microscopy technologies, integrated them, and demonstrated their workability to perform concurrent electrophysiology and two-photon microscopy experiments, no formal experiments have yet been conducted. The work performed in each thrust has furthered the understanding and capability of performing concurrent long-term electrophysiology and two-photon microscopy experiments.

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