CHARACTERIZATION OF NEURITE GROWTH ON A NOVEL NEUROTUBE PLATFORM

BY

OLIVIA VASSILIKI CANGELLARIS

THESIS

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Advisor:

Professor Martha U. Gillette
ABSTRACT

The dichotomy between the soul and the body, the mind and the brain, has puzzled philosophers and scientists alike since the ancient Greeks. Despite enormous advances in science, engineering, and medicine, we are still working to understand one of the greatest mysteries: the brain. For years, neuroscientists, biologists, physicists, and engineers have been investigating neurons in a variety of ways in order to fully understand how they function and organize into the complex structure of the brain. We know that electric fields play a critical role in the development of organisms in vivo, especially with respect to neural tube formation, and regeneration; the ability to guide proliferation and migration of neurons in vitro is being continually studied: multi-electrode arrays allow for the development and maintenance of functioning neural networks that can be programmed to complete a task; and yet, after ninety years of exploring electrical dimensions of tissue slices and cultures, there is still a wealth of information yet to be deciphered. In particular the role of dendrites in network formation and communication was largely overlooked while the axon was considered the principal player. Dendrites and their spines, which are the site of memory formation, play a pivotal role in learning and memory. This thesis introduces a novel method for investigating, at the single cell level, the response of the dendrites of hippocampal neurons to applied electric fields. Initial cultures on an array of biocompatible, optically transparent, self-rolling microtubes are presented and the interaction of the neurites with the microtubes is analyzed. This novel substrate and application have the potential to provide unique insights into the mechanisms that drive organization of the dendritic architecture of hippocampal neurons, and may prove valuable in investigating new methods of treating neurological and developmental disorders.
Dedication

To the memory of my aunt and godmother, Antonia, whose love and support is eternal.
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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW AND SIGNIFICANCE

The dependence of neurons on chemical cues as a determining factor in neurite outgrowth and network formation has been studied extensively. Semaphorin 3A guides dendrite growth, and subsequently affects a neuron’s polarity [1], [2]; laminin can be used to selectively guide axons [3], [4]. It is apparent that chemical signaling plays an important role in neural network formation. However, in addition to chemical signaling, an abundance of electrical activity occurs in the brain. There are many instances in which endogenous electric fields (EFs) are produced in vivo. For example, during development of Xenopus embryos, specific voltage potentials were measured in the eye fields and play a crucial role in specifying where the eyes will develop; when these potentials were mimicked elsewhere in the embryo, ectopic eyes formed at the manipulated locations as well [5].

Not only are endogenous fields abundant in vivo, but EFs have been shown to affect in vitro systems as early as 1920 when Sven Ingvar demonstrated that applied DC fields direct the outgrowth of processes in a neuron culture [6]. Since then, electrical behaviors of cells have been studied in a variety of ways, including patch clamping, galvanic cells and salt bridges, and, beginning in the mid-1970s, through the use of multi-electrode arrays (MEAs). The focus has gradually shifted to solidifying our understanding of neural network formation. From an engineering perspective, the key to unlocking the secrets of the brain lies in our ability to understand, fundamentally, how neurons self-organize to produce these complex networks.

Although significant progress has been made in maintaining neural networks on MEAs and designing flexible MEAs that can interface with the brain itself, the questions concerning what decisions are being made at the subcellular level, and the critical role that dendrites play, still remain [7]–[9].

When working with complex neural networks, it is extremely difficult to isolate the behavior of a single cell from the entire network. Through the use of low-density cultures on a novel platform designed by Professor Xiuling Li’s research group in
Electrical and Computer Engineering at the University of Illinois, Urbana-Champaign, it is possible to interrogate the response of a single neuron to EF effects. The platform holds an array of neurotubes (NTbs), which are highly customizable rolled silicon-nitride membranes: the thickness, length, porosity, and separation distance can all be easily adjusted [10]. These NTbs can be functionalized with electrodes and powered so that electric fields are generated on the platform. The work presented in this thesis lays the foundation for the study of EF effects on the dendritic architecture of hippocampal neurons using this novel substrate. The overarching goal is to characterize how the EF impacts dendritic development, which is dependent upon the subcellular response at the level of the dendritic filopodia, guides the motility of neurons, and facilitates the development of their complex architecture.

1.2 ORGANIZATION OF THESIS
This thesis is organized into five chapters. Chapter 1 provides a brief overview and discussion of the significance of this work. Chapter 2 provides concise, yet fundamental information about the neuron, the hippocampus, and neuronal development. Additionally, it includes a broad review of the literature most relevant to this project including: global application of electric fields in vitro, a brief history of multi-electrode arrays, and the introduction of a new platform for manipulation of neurons with electric and magnetic fields. Chapter 3 details the various protocols and materials used throughout this study: substrate fabrication, neuron cell culture, fixation and staining, immunofluorescent imaging, SEM imaging, and image processing and analysis. Chapter 4 contains preliminary results of this project and a discussion and interpretation of the data. Chapter 5 considers the potential of this work, how the project will advance, and the clinical implications of this emerging method.
CHAPTER 2: BACKGROUND

2.1 WHAT IS A NEURON?

Neurons are specialized cells that play fundamental roles in the central and peripheral nervous systems (CNS and PNS, respectively). There are many distinct types of neurons, depending on their location in the CNS or PNS, and their function [11]. However, despite their diversity, the organization of a neuron can be distilled into three common features: a soma, or cell body; a single axon; and a complexly branched dendritic tree. The simplified structure is illustrated in Figure 1.1.

![Fundamental structure of a neuron](image)

**Figure 2.1: Fundamental structure of a neuron**

Within the brain, neurons establish networks by which they communicate with each other, and process and act on information retrieved from other organs in the body or the external environment. Neurons connect to each other via the synapse: a site of communication between two neurites from two different cells. A synapse can form between two dendrites: dendro-dendritic; between two axons: axo-axonic; between an axon and a dendrite: axo-dendritic; or even between an axon and a soma: axo-somatic [12]–[14]. In Figure 1.2, we consider the interaction between an axon and a dendrite. In this case, Neuron 1 has generated a signal that is propagated along its axon to the synapse. Signals of sufficient amplitude cause release of chemical signals from Neuron 1 that diffuse across a 20 nm gap to Neuron 2. The dendrite of Neuron 2 receives this signal via receptors that alter the electrical state of the membrane, and sends it toward the soma of Neuron 2. The change in membrane excitability is integrated with other
signals from other dendrites and may reach the soma where it may activate the axon and cause changes in cellular processes in the soma. Synapse formation is critical for the greater organization of neural networks.

![Figure 2.2: An axo-dendritic synapse (*)](image)

(a) Signal generated at soma of Neuron 1; (b) Signal propagated along axon of Neuron 1; (c) Signal transfer at synapse; (d) Signal propagated along dendrite of Neuron 2; (e) Signal processed in soma of Neuron 2.

### 2.1.1 Ion Channels and Electrical Signals in Neurons

The signaling between neurons described in the previous section depends on the movement of ions into and out of the cell, and the diffusion of neurotransmitters (NTs) across the synapse. Ions are shuttled across the cell membrane through ion channels, proteins that span the membrane of the cell, of which there are two main classes: (1) ligand-gated; (2) voltage-gated. The ligand-gated ion channels allow ions to pass through the cell membrane; they open when bound by a specific chemical messenger, or ligand. Examples of ligand-gated ion channels include nicotinic-acetylcholine receptors, which bind the NT acetylcholine [11].

The most well-recognized ion channels are probably the voltage-gated channels, including the Na\(^+\) and K\(^+\) channels, which are crucial for maintaining the resting
membrane potential of a neuron, and for the generation of action potentials. They are activated by changes in the membrane potential.

The resting membrane potential \(V_{\text{rest}}\) of a neuron is about -70 mV, with respect to the outside of the neuron. The internal concentration of Na\(^+\) \([\text{Na}^+]_i\) is low, with respect to the external concentration \([\text{Na}^+]_o\), \([\text{K}^+]_i > [\text{K}^+]_o\), and \([\text{Cl}^-]_i < [\text{Cl}^-]_o\). The \(V_{\text{rest}}\) is maintained by the \(\text{Na}^+,\text{K}^+-\text{ATPase}\) pump, which is an ionic pump that sends 2 Na\(^+\) ions out of the cell and shuttles 3 K\(^+\) ions into the cell through the conversion of ATP (adenosine triphosphate, the energy currency of cells) to ADP (adenosine diphosphate) + Pi. Without the ion pump, the concentration gradients would overwhelm the cells and Na\(^+\) and Cl\(^-\) would flow down their concentration gradients into the cell to equilibrate, while K\(^+\) would flow down its concentration gradient out of the cell to equilibrate, and the cell would die.

In addition to the concentration gradients and the ATPase pump, there are voltage gradients established by the difference in potential of the inside of the cell relative to the outside. Because the inside of the cell is more negative than the outside, the positive K\(^+\) are encouraged to flow inside the cell, against the concentration gradient, but along the voltage gradient. The voltage gradient is what counteracts the diffusion of K\(^+\) out of the cell. Na\(^+\) and Cl\(^-\) also have voltage gradients.

During the generation of an action potential, the permeability of each ion channel changes such that they open and the ions can flow steadily down their concentration gradient. An action potential begins when the neuron receives a stimulus strong enough to depolarize its membrane above -55 mV. The Na\(^+\) channels are activated and open first, allowing Na\(^+\) ions into the cell, which further depolarizes the membrane, leading to the activation and opening of the K\(^+\) channels. The Na\(^+\) channels are then inactivated and the cell begins to repolarize, however the K\(^+\) channels do not close right away and this delay leads to a hyperpolarization of the membrane, causing the cell to enter the refractory period. Once the K\(^+\) channels close, the membrane returns to the resting state [11].

2.2 THE HIPPOCAMPUS

Within the brain, nestled beneath each temporal lobe in the cerebral cortex, lies the hippocampus. The hippocampus is composed of two regions: the dentate gyrus and
the cornu ammonis, with two distinct neuron types. The dentate gyrus consists primarily of granular cells, while the remainder of the hippocampus is composed primarily of pyramidal cells [15]; however in culture, the whole hippocampus is isolated and used (Figure 2.3). A structure of the limbic system, the hippocampus is known to play a critical role in learning and memory [16]–[18]; it is a brain-site whose cells and functions are altered in developmental and neurodegenerative disorders [19]–[21]. The neurons in the hippocampus are a desirable model for studies involving network formation because pyramidal neurons form extensively branched dendrites with numerous stabilized spines [22], [23]. Because these neurons are critical for memory formation, their role is a focal point for research intent on deciphering how information is processed within a neural network.

Figure 2.3: Hippocampal brain slice from a rat. Cell nuclei stained with DAPI, a nuclear marker (white staining). Regions of the hippocampus marked: Dentate Gyrus (DG), Cornu ammonis regions 1 and 3 (CA1 and CA3). Photo courtesy of Mia Yu.

2.3 DENDRITE DEVELOPMENT AND FILOPODIA

Before a neuron develops its complex dendritic network, the dendritic tree is composed of nothing more than a dendritic arbor with a few primary branches, each of which terminates in a dendritic growth cone (Figure 2.4a). The second stage of
development is known as synaptogenesis (Figure 2.4b). During this stage, the arbor and primary branches sprout fine, highly dynamic structures known as filopodia [24].

A filopodium is an extension of the cell membrane and cytoskeleton that is capable of rapid formation, elongation, and remodeling. Filopodial dynamics have been observed in both hippocampal tissue slices and dissociated cultures of hippocampal neurons. They have been shown to grow as much as 5-10 μm in a single minute with a lifetime of about 10 minutes [25], [26]. True to their rapid growth and turn over, the filopodia actively search out the environment surrounding their associated neuron, sensing signals and determining its fate. Some filopodia search for a target axon with which to form a synapse; others choose to extend, branch out and produce new processes; and some filopodia will stabilize into dendritic spines [24]. Filopodia play a critical role in memory formation and recall, since the creation of memories depends on the stabilization of dendritic spines [27], [28]. At the end of synaptogenesis, a mature dendritic tree has an extensively branched network of dendrites, which lack filopodia. In

![Figure 2.4: A pictorial representation of the three stages of development of the dendritic arbor. In (a), (b), and (c), the bottom figures are an enlargement of the dendritic shaft and growth cone and filopodial interactions. (a) The soma with the dendritic arbor and a limited number of primary branches. Typically, an axonal growth cone (agc) can be found in the vicinity of the developing arbor and its dendritic growth cones (dgc). (b) The developing arbor as it goes through the process of synaptogenesis. Dendritic filopodia (df) are shown emanating from the dendritic shaft. (c) The mature neuron with its extensive dendritic tree. Stabilized spines (sp) have formed connections with the neighboring axon. (Wong et al. 2000) [24]
lieu of a filopodium, a dendritic spine is often found, sometimes with an accompanying synapse, as illustrated in Figure 2.4c [24].

Importantly, filopodial dynamics are actin-driven in response to extracellular signals. Even if a neuron is not electrically active, the filopodia will still grow. Dunaevsky et al. demonstrated these findings when they applied Cytochalasin D, an actin polymerization inhibitor, to cultures of pyramidal neurons and showed that spine motility was suppressed. However, in the absence of sodium, rendering sodium currents non-existent in the culture, and by blocking synaptic activity with L-2-amino-5-phosphonovaleate/6-cyano-7-nitroquinoxaline-2,3-dione, there was no noticeable change in the dendritic spine motility [29]. Therefore, even neurons that are relatively isolated from other neurons in a culture undergo this early development.

The transition from filopodium to either a stabilized spine or development into a longer branch is dependent upon cues found in its environment. There are a number of signals that filopodia respond to, including extracellular NTs and intracellular calcium transients [24], [30], [31]. The role of NTs in stabilization of filopodia can be extrapolated from the combined results of several studies performed on neurons from the midbrain of Xenopus that showed restricted growth of dendrites following: (1) the application of an NMDA receptor antagonist to the cultures, which blocks the ability of the synapse to be activated by the NT glutamate [32]; (2) the interference with or blocking of GABA or AMPA receptors, thereby inhibiting the post-synaptic response to NTs [33], [34]. Calcium transients may be critical elements of filopodial stabilization. Lohmann et al. demonstrated that diminished calcium transients lead to an increase in filopodial growth, but increased calcium transients result in stabilization of existing filopodia and hinder the growth of new filopodia [35]. Furthermore, through time-lapse imaging, Lohmann et al. showed that upon contact between a filopodium and an axon, the local calcium transients tripled in frequency and this increase in frequency anticipated the stabilization of the filopodium [36].

In addition to filopodial dynamics, which are important contributors to synapse formation and initial growth of dendritic processes, the response of dendrites themselves to external cues helps to shape the development of the dendritic tree. Signals that have been shown to affect dendrite behavior include chemical cues, which can selectively guide dendrites, as is the case with Sema3A [1], [2], [37], while laminin
preferentially selects for axons, discouraging dendrite growth [3], [4]; as well as topographical cues such as nanometer scale ridges or valleys [38], [39]. In addition to these cues, electrical signals are also important for dendrite development.

2.4 ELECTRIC FIELDS AND IN VITRO CULTURES

Neurons are *electrically excitable* cells. It is easy to overlook the presence of EFs in the brain if the focus remains on the movement of NTs, and the shuttling of ions across the neuron. But ions are charged and the movement of charges creates an EF. As mentioned in Chapter 1; endogenous EFs are found *in vivo*, and the electrical activity of neurons has been studied for over ninety years through a variety of electrophysiological methods, the manipulation of voltage gradients *in vivo*, and the study of global EFs applied across *in vitro* systems.

2.4.1 ELECTRICAL SIGNALS ARE CRITICAL FOR DEVELOPMENT

Numerous studies involving *Xenopus* and chick embryos have measured steady currents that flow through different parts of the embryo at different stages of development. These currents generate voltage gradients, which change in location and magnitude over the course of development as various components of the animal’s anatomy, especially the neurotube from which the CNS develops, are formed [40]–[44]. This endogenous electrical activity is referred to as ‘bioelectricity’ and is essential not only for early development, but also recovery from tissue injuries, regeneration of limbs, and the proliferation, differentiation, and even programmed death (apoptosis) of cells [45]–[51].

Michael Levin has demonstrated the critical role of bioelectricity in development with a series of experiments that show how altering the voltage gradients can be used to direct the formation of organs, and disrupt anatomy on a macro-scale. His manipulation of the voltage gradients is achieved by altering the resting membrane potential ($V_{\text{mem}}$) through genetic manipulation in order to control the expression of certain ion channels. The approach was to inject mRNAs with a dominant-negative construct for two subunits of the ATP-sensitive potassium channel ($K_{\text{ATP}}$), which is important for $V_{\text{mem}}$ association with cell function [52] and known to be expressed in the eye fields of *Xenopus laevis*) into the embryos. The Levin group showed that at stage 42 the tadpoles expressed a
whole ectopic eye complete with a lens caudal to the normal eye on the right side, as well as ectopic eye tissue in other parts of the head and in the tail region [5]. They were also able to induce the formation of an ectopic beating heart through misexpression of ion channels. The Levin group has also demonstrated the ability to induce regeneration of amputated Xenopus limbs even though this regeneration is unnatural (Figure 2.5) [53].

![Control](image1)

**Figure 2.5: Controlled regeneration of frog hind limb following amputation.** One hind limb of each tadpole was amputated at the location of the tibia-fibula, then 1 day post-amputation (dpa) the control tadpoles were treated with 0.1% Ethanol and the experimental tadpoles were treated with a cocktail made up of 20 µM Monensin and 90 mM Na-Gluconate in 0.1 Mark’s Modified Ringer’s (XMMR), for 1 hour. The tadpoles matured to 45 dpa. (a) Section of control limbs show no up-regulation of the regenerative marker MSX-1 (white arrowhead; yellow arrowhead indicates other cells expressing MSX-1); (b) Section of cocktail-treated limbs shows MSX-1 positive region (green arrowhead; yellow arrowhead indicates other cells expressing MSX-1); (c) Control limb 45 dpa shows little to no growth beyond amputation site; (d) Experimental limb 45 dpa shows regrowth beyond the amputation site, including toes and toenails (green arrowheads) suggesting genuine morphogenesis. In all experimental cases, a limb was regenerated. (Adapted from Tseng et al. 2013) [53]

### 2.4.2 GLOBALLY APPLIED ELECTRIC FIELDS IN VITRO

These *in vivo* studies classifying the role of EFs and voltage gradients are a defining branch of the field of study involving electrical control of cellular behavior. In addition to *in vivo* studies, the investigation of EF effects on cultures has been explored for decades, and is critical for elucidating the mechanism of the response that leads to
proliferation, migration, and general growth at the cellular level, particularly when it comes to neurons, given the complexity of the brain, CNS, and PNS.

Many of the experiments conducted with EFs applied globally to cultures have been conducted using a specifically designed chamber connected to a DC power supply via agar salt bridges and Ag/AgCl electrodes, in order to approximate as faithfully as possible the endogenous EFs found in vivo (Figure 2.6).

In 1920, Sven Ingvar announced that processes from chick brain tissue in culture extended in line with the galvanic field lines and that the morphology of the processes growing towards the anode was different from those growing towards the cathode. By 1946, evidence of this phenomenon was published by Marsh and Beams in which they showed chick medullary neurites growing towards the cathode in the presence of a 50-60 mV/mm EF; this study was further verified and quantified in 1979 by Jaffe and Poo in which the neurites from the chick dorsal root ganglia (DRG) were shown to grow three times faster when growing in the direction of the cathode, compared to the anode, in the presence of an EF >70 mV/mm [46], [54]. This preference of the neurites for the cathode, and their increased rate of growth in that direction is true of spinal neurons from Xenopus as well, and processes facing the anode have been shown to reabsorb into the soma when left in the presence of the EF for two hours [55].

Since these early experiments, further in vitro studies involving neurons from Xenopus, chicks, lampreys, and rats, among others, have yielded several findings
including: (1) EFs direct neurite growth generally toward the cathode, although different types of neurons have shown varied results (processes from motor neurons will turn toward the cathode, but processes from sensory neurons do not), and dendrites in embryonic rat hippocampal neurons are drawn to the cathode, while the axon is not [54], [56], [57]; (2) processes nearer to the cathode exhibit increased branching, while those facing the anode are resorbed [55], [58], [59]; (3) rat hippocampal neurons have been shown to migrate in the direction of the EF (moving from the anode towards the cathode) when cultured in the presence of a 120 mV/mm EF (within the physiological range of endogenous EFs) [60]; and, (4) the time it takes for regeneration of axons in transected spinal cords of lampreys is reduced when a DC EF is applied with a polarity that opposes the injury potential [61], [62].

2.4.3 MULTI-ELECTRODE ARRAYS

Shortly before McCaig, Borgens, and others began to study how EFs impacted neuronal development in vitro, Charles A. Thomas et al. published a paper describing a multi-electrode array (MEA) that could be used to record signals from cultured tissue slices or dissociated cells. This first MEA was fabricated on glass and consisted of two rows of 15 7-µm-square platinum black-plated gold electrodes, with a separation distance of 100 µm, insulated by photoresist. The first successful recording from this device came from dissociated chick myocytes; the signal recorded was the contraction of the myocyte layer that had formed [63]. The second successful recording came from a unique MEA developed by Guenter Gross et al. and consisted of set of 36 electrodes with a 10 µm diameter, a 100-200 µm separation distance, and a thermosetting polymer as the insulator. Gross et al. were able to record from a single snail ganglion [64]. The third unique MEA to be introduced came in 1980 from Jerome Pine and this was the first device from which dissociated neurons (superior cervical ganglia from a rat) yielded recorded signals [65].

Between 1970 and the early 2000s, a number of research groups worked to improve upon the MEA platform by fine-tuning the fabrication methods and cell culture parameters [66]. The goal of the MEA was to provide a substrate upon which neuron cultures or brain slices could be cultured successfully over long periods of time without diminished health, and simultaneously be exposed to relatively constant electrical
signals. The other desire was to be able to record signals from the neurons. With these three constraints satisfied it would be possible to explore the functionality of neural networks and what drives their development. One of the major advantages of MEAs, over patch-clamping in particular, is that they are non-invasive (although some MEAs are designed to integrate patch-clamping techniques with the MEA platform). Steve Potter and Thomas Demarse were able to maintain an MEA culture of rat cortical neurons alive for 15 months (Figure 2.7) [67].

![MEA image and recording of 15 month-old rat cortical neuron culture.](image)

**Figure 2.7: MEA image and recording of 15 month-old rat cortical neuron culture.** (Top) A phase contrast image of a 15 month-old culture of rat cortical neurons. The somas are largely obscured by glia and fascicles. Electrodes and leads are in black. Scale = 200 µm between electrodes; (Bottom) Spontaneous activity of the network, recorded after 1 year in culture using MultiChannel Systems MEA60. Each dot corresponds to an action potential recorded by one of the MEA channels. (Adapted from Potter et al. 2001) [67]

The Potter lab has had notable success with the MEA platform and accompanying software. His group wrote a closed-loop, open source program called NeuroRighter that couples to a multichannel hardware platform, which can stimulate and record from up to 64 channels [68]. They also developed MEART (Multi-Electrode Array Art), a drawing robot with two pneumatic arms coupled, via the internet, to an MEA culture of rat cortical neurons in Potter’s lab at GeorgiaTech, as far as 12,000 miles away. The arms held pens and were suspended over a piece of paper. Based on the output from the network of cortical neurons, MEART would move the pens across the paper to create a work of art. The goal for MEART was to draw a 12 x 12 cm solid square within a larger 30 x 30 cm area. A CCD camera captured the image drawn by the arms, converted it to a pixel format, and transmitted this information back to the network, thereby providing sensory feedback. MEART did not demonstrate successful learning, however, it did demonstrate neural plasticity [69].
One of the drawbacks to MEAs is the high density at which cells are seeded, and the dense networks of neurons that subsequently form. Although MEAs technically make it possible to stimulate and record from a soma or process, the information that is obtained is potentially confounded by multiple connections to that process or soma. As a result, if the question meant to be answered is what response external electrical signals elicit from a single neuron, or what signals a single process generates in response to a stimulus of interest, and this is to be independent of the complexity conferred by neighboring cells, then an MEA is arguably not a robust enough substrate.

One way to overcome this limitation is to couple MEAs to microfluidic devices (µFDs). Several research groups have documented µFDs integrated with an MEA. These devices include: (1) an MEA with PDMS microtunnels through which axons grow and can be stimulated or recorded from [70]; (2) an MEA with PDMS mini-wells in which hippocampal brain slices are cultured; this platform also contains microchannels through which axon projections can grow and be studied [71].

2.5 A NEW PLATFORM FOR DIRECTED STIMULATION OF IN VITRO SYSTEMS

McCaig suggested that since the advent of microelectrodes for intracellular recording and patch-clamping, the focus on global effects of EFs and the resultant changes in the cell’s extracellular voltage signals shifted: the new interest became the study of transmembrane potentials and single ion currents [6]. These research endeavors are unquestionably valuable, however with these methods, the response of the cell beyond the behavior of a single ion channel is lost. Even with MEAs, the information from a recording electrode is complicated by the fact that a single neuron is connected to many other cells. Therefore, even if that one neuron of interest is being recorded from, distilling the recorded response to a single neuron proves difficult and there is a strong possibility that the recorded response has been influenced by other neurons in the network. Integration of MEAs with microfluidic devices for the purpose of exploring the behavior of neuronal processes has been limited and largely restricted to the study of axons. In fact, the understanding of the dendritic response to externally applied EFs is lacking. In order to facilitate the continued exploration of EF effects on
dendrites of hippocampal neurons, with the goal of elucidating critical aspects of the role of these cues in development, a different type of substrate is necessary.

A substrate, consisting of an array of thin silicon nitride films that are strained such that the membranes self-roll into three-dimensional tubes, was designed by Froeter et al. [10]. These microtubes, termed neurotubes (NTbs) for their application to neuron culture, are biocompatible: the substrate consists of a glass base, the silicon nitride (SiN_x) membranes, and a silicon dioxide anchor. NTbs are optically transparent to facilitate imaging of both live and fixed samples [10]. Additionally, the membrane of each NTb can be designed so that the diameter is no larger than 5 µm, on average, therefore preventing the infiltration of the soma, while allowing either the dendrite or axon to grow through the NTb.

Notably, the inner membrane of the NTb can be patterned with electrodes prior to rolling so that the electrodes end up on the inner diameter of the NTb. The NTbs can be functionalized as conductors, inductors, or capacitors, this allows for the selective stimulation of a process within the powered NTb with electric or magnetic fields. It is also possible to record signals from the NTbs. Coupling low-density cultures to this novel platform with the ability to selectively power regions of the array should prove to be an invaluable tool for investigating the intrinsic fields associated with hippocampal processes, especially dendrites, and characterizing the effect applied fields have on neuronal development at an intimate level.
Figure 2.8: Scanning Electron Microscope (SEM) images of silicon nitride-based hierarchical hybrid neurotubes (NTbs). (a) Inductor design with four turns of 5 nm Ni/60 nm Au/5 nm Ni square wave pattern, rolled-up by a 36 nm strained SiN₉ bilayer; (b) 16 nm LF SiNₓ/20 nm HF SiNₓ/7 nm Cr (exposed)/70 nm Au quadlayer rolled downward due to local stress from chromium oxide. (Adapted from Froeter et al. 2013) [10]
CHAPTER 3: METHODOLOGY

3.1 NEUROTUBE SUBSTRATE

The neurotube (NTb) platform used for the experiments presented in this thesis were designed, developed, and fabricated by Paul Froeter, in Professor Xiuling Li’s research group. The following description of the devices is detailed with their permission and adapted from Froeter et al. 2013. Further detail concerning the fabrication can be found in the Methods section of that paper [10].

3.1.1 DEVICE FABRICATION

Each NTb device consists of five layers: the substrate, a sacrificial layer, a layer of low frequency (LF) silicon nitride (SiNx), a layer of high frequency (HF) SiNx, and a SiOx anchor. The substrate is a silicon dioxide (SiO2) #1.5 cover slip (0.16 - 0.19mm thick), and the sacrificial layer is germanium (Ge). The first layer of SiNx, applied at LF has a compressive strain, while the second layer (HF SiNx) has a tensile strain. This difference in strain is critical for the self-rolling of the NTbs [10].

Following a standard solvent clean (acetone, isopropyl alcohol (IPA), deionized water (DI), IPA) and N2 dry, a layer of germanium is deposited on the substrate using E-beam evaporation. Next, the two layers of SiNx are deposited using plasma-enhanced chemical vapor deposition (PECVD) of a NH3/SiH4/N2 gas mixture. By varying the RF power and frequency, as well as the pressure, the intrinsic strain in SiNx can be tuned to yield either compressive or tensile films. The compressive strain is achieved by using a higher power, lower frequency, and higher pressure than those conditions for the tensile strain. The primary reason for the large difference in strain is plasma operation frequency, which causes a significant density difference [11].

After the two layers of SiNx are added to the substrate, a layer of positive photoresist (PR) is spun onto the wafer. Using photolithography and a mask, which designates and protects the pattern of the SiNx array, the PR is exposed to UV light. The wafer is then dry etched using Freon 14 (CH4) reactive ion etching (RIE). The SiNx covered by the PR is protected from the etchant, while the exposed SiNx, as well as the underlying sacrificial layer is eaten away. Finally, the PR is removed using acetone and
the result is a device with islands of stacked Ge, LF SiN$_x$, and HF SiN$_x$, surrounded by SiO$_2$. At this point, if needed, the SiO$_x$ anchor is deposited on one edge of the island, using a photolithography lift-off technique. Finally, the sample is submerged in 30% H$_2$O$_2$, removing the germanium and rolling the NTbs. Finally, the device is dipped in methanol and subsequently dried on a hot plate set to 110°C.

![Diagram of NTb Device Flow Process.](image)

**Figure 3.1: NTb Device Flow Process.** Begin with a SiO$_2$ substrate; deposit a sacrificial layer of Ge; PECVD deposition of LF SiN$_x$ and HF SiN$_x$; spin coat photoresist; photolithography exposes areas of SiN$_x$/Ge to etch; RIE with CH$_4$ to remove SiN$_x$ and sacrificial layer; remove residual photoresist; deposit SiO$_x$ anchor; 30% H$_2$O$_2$ lateral etch to remove Ge below pads and allow SiN$_x$ membrane to roll. Adapted from Froeter *et al.* 2013 [10].

### 3.1.2 Device Characteristics

For the experiments detailed in this thesis, two different NTb geometries were used, classified as either *array* or *helical*. Within the array category, two devices were fully etched (FE), meaning the sacrificial layer beneath the SiN$_x$ pads was completely removed. One FE device had a small yield, only 20% of the NTbs were intact before culture, and one FE device had a large yield, almost 100%. The % yield is determined by counting the intact and broken NTbs in one 5 x 5 mm area along the perimeter of the 1 x 1 cm sample, since the perimeter is more variable than the center. In the case of the 20%
array, the yield was diminished because the SiO$_2$ anchor was placed 6 µm into the SiN$_x$ membrane so the NTbs only rolled through a $\frac{3}{4}$ turn during the etch. The membrane is 17 µm before the anchor is deposited, and in order for the NTb to make one complete turn, the membrane must have at least 14 µm through which to turn. By depositing the sample at 6 µm into the membrane, as opposed to the standard 3 µm, the resultant length of the membrane was 11 µm. As the sample was dried, the capillary forces unrolled some of the tubes, leading to a low yield. The other array device was partially etched and fully collapsed (PEFC), meaning some sacrificial Ge remained surrounding the SiN$_x$ pad (PE) and the tubes remained unrolled (FC). The spacing between NTbs on the array substrates was 40 µm, and the length of each NTb was 50 µm.

Within the helical category, there were two devices: NTb-helical and NTb-ghelical. In these devices, the SiN$_x$ pattern is shaped like a ladder, and the membrane roles in a helical fashion. The NTb-helical has no exposed sacrificial layer, while the NTb-ghelical has some remaining sacrificial layer beneath the membrane. The membrane of the helical NTbs includes multiple turns (4), while the membrane of the array NTbs turns only once to form a single rolled microtube.

![Figure 3.2: Examples of Array and Helical NTbs. (a) Depicts an array of single rolled NTbs with a diameter of 4.4 µm, inset depicts a single NTb. (b) Depicts a single, rolled helical NTb. (Adapted from Froeter et al. 2013) [10]](image)

The following conditions were constant for each NTb substrate condition: the thickness of the SiN$_x$ membrane was 40 nm; the diameter of each NTb ($D_{NTb}$) was approximately 4.5 µm; the SiO$_2$ anchor was 100 nm thick.
3.1.3 CONTROLS AND SUBSTRATE PREPARATION FOR CELL CULTURE

As a control, neurons were cultured on a SiO$_2$ coverslip. Additionally, to account for the presence of SiN$_x$ on the NTb platforms, a second control was fabricated, consisting of layers of SiN$_x$ deposited on a SiO$_2$ coverslide, one layer of LF SiN$_x$ at a thickness of 20 nm, and one layer of HF SiN$_x$ also at a thickness of 20 nm. This control is referred to as the planar SiN$_x$ control (pSiN$_x$).

Prior to cell culture, each substrate was moved to a biosafety cabinet and placed in a sterile 35 mm tissue culture dish (Corning). Its surface was covered with sterile-filtered poly-D-lysine (PDL, 100 µg mL$^{-1}$, Sigma-Aldrich) and the PDL was allowed to adsorb to the surface for one hour before the excess PDL was aspirated from the surface, and the substrate was then allowed to air dry.

3.2 CELL CULTURE

Primary hippocampal neurons were harvested from postnatal day two (P2) Long-Evans BluGill rats, a genetically homogeneous inbred line developed by the Gillette lab at the University of Illinois at Urbana-Champaign. The animals were used according to the protocols established by the University of Illinois Institutional Animal Care and Use Committee, as well as all state and federal regulations.

3.2.1 MATERIALS

Hibernate-A (Brain-Bits, Springfield, IL) and Neurobasal-A (Invitrogen) media, without phenol-red, were each supplemented with 0.25% GlutaMAX™ (Invitrogen), 2% B-27 (Invitrogen), and 1% 100 U mL$^{-1}$ penicillin and 0.1 mg mL$^{-1}$ streptomycin (Sigma-Aldrich).
3.2.2 HIPPOCAMPAL DISSECTION AND DISSOCIATION

The rat’s head was swiftly decapitated with scissors, then the cerebrum sans frontal lobe was removed to a 35 mm tissue culture dish (Corning) on ice, and submerged in 4°C Hibernate-A. Two hippocampi were removed from beneath each cortex of two rats, and a total of four hippocampi were placed in a 15-mL conical tube containing Hibernate-A with activated papain (25 U mL⁻¹, Worthington) for 15 min at 37° C. The conical tube was gently agitated every 5 min to ensure separation of the hippocampi such that each hippocampus was completely exposed to the enzyme, for optimal results.

Following the enzyme treatment, the conical tube was moved to a biosafety cabinet, the papain solution was aspirated, and the tissue was washed with enzyme-free Hibernate-A. Next, 2 mL of fresh Hibernate-A were added and the cells were mechanically dissociated via trituration with a fire-polished glass Pasteur pipette. Once the undissociated tissue was allowed to settle, the supernatant containing cells being dissociated was removed to a new 15 mL conical tube and the trituration was repeated with 1 mL of Hibernate-A. Then, the enzyme treatment, wash, and mechanical dissociation were repeated.

The combined cell suspension was centrifuged at 1400 rpm for 5 min. The supernatant was then aspirated and the pellet resuspended in 1 mL of Neurobasal-A. Live cells were counted using a hemocytometer and Trypan Blue exclusion (0.4%, Invitrogen). The cells were seeded at a density of either 150, 200, or 600 cells mm⁻² (specified within Chapter 4). 500 µL of cell suspension was added to the area of either a control SiO₂ coverslide, a coverslide with 20 nm HF/20 nm LF pSiNx, or the NTb substrate (preparation described above), and allowed to sit for 30 min in the incubator to allow the neurons to attach to the substrate, then an additional 1.5 mL of fresh Neurobasal-A was added to each dish. The cells were kept in a humidified incubator at 37° C with 5% CO₂ for four days before fixation.

3.3 FIXATION AND IMMUNOCYTOCHEMISTRY

After four days in vitro (4 DIV), the cultures were removed from the incubator and fixed with 4% Electron Microscopy grade (EM-grade) paraformaldehyde (16% paraformaldehyde, Electron Microscopy Sciences, diluted to 4% in de-ionized water
from a Milli-Q Ultrapure Water System) for 20 min on a shaker. Then, the cells were washed 4 times for 30 sec with phosphate buffered saline (PBS, pH 7.4). A fifth wash was left on for an additional 5 min. This complete washing sequence was repeated between every subsequent solution exchange, except between the blocking and primary antibody. Next, the cells were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, diluted in PBS) for 10 min and blocked with 5% normal goat serum (NGS, Sigma-Aldrich, diluted in PBS) for 30 min. A 2% NGS solution was prepared for each antibody.

The cultures were stained with the following primary antibodies: rabbit polyclonal anti-MAP2 (1 : 1,000, Chemicon) to label the dendrites, and mouse mono-clonal anti-GFAP (1 : 1,000, Chemicon) to identify the astrocytes. Once the primary antibodies were added to the culture dishes, they were incubated at room temperature for one hour on a shaker, and then moved to a refrigerator and incubated overnight at 4° C. The following day, the cells were washed for 10 min in PBS, then the secondary antibodies were added and incubated at room temperature for one hour on a shaker. The secondary antibodies were: Alexa Fluor 594 goat anti-rabbit (1 : 1,000, Molecular Probes); Alexa Fluor 488 goat anti-mouse (1 : 1,000, Molecular Probes). Finally, the cells were stained with DAPI (1 : 50,000, Invitrogen), a nuclear marker, for 10 min at room temperature. The cells were washed for 10 min with PBS, stored in fresh PBS, and then imaged by immunocytochemistry (ICC).

3.4 IMMUNOFLUORESCENT IMAGING

ICC imaging was performed on a Zeiss Axiover 100 TV fluorescence microscope at 10x and 40x. A few cultures on the NTb substrates were imaged on a Nikon Optiphot-2 fluorescence and phase-contrast microscope at 10x. Following fluorescent microscopy, a subset of the NTb substrates was imaged by scanning electron microscopy (SEM).

3.5 SCANNING ELECTRON MICROSCOPY

3.5.1 PREPARATION

The cultures designated for SEM imaging were first re-fixed with 2.0% E.M. grade paraformaldehyde (Sigma-Aldrich) and 2.5% E.M. grade glutaraldehyde (Sigma-Aldrich) in 0.1 M Na-Cacodylate buffer (Sigma-Aldrich, pH 7.4) for four hours at 4° C. Then the cells were rinsed for 10 min on a shaker in 0.1 M Na-Cacodylate buffer.
Following the buffer rinse, the cells were dehydrated in 37% ethanol (EtOH), 67% EtOH, 95% EtOH, and three 100% EtOH washes for 10 minutes each, on a shaker. Following the EtOH dehydration, the samples were brought to the critical dry point in a Tousimis Samdri-PVT-3D critical dryer. Finally, the samples were coated with 10 nm of Au/Pd using a Denton Vacuum Desk-1 TSC sputter coater.

### 3.5.2 IMAGING

The processed substrates were imaged on the environmental SEM (ESEM) in the Microscopy Suite at the Beckman Institute at the University of Illinois at Urbana-Champaign.

### 3.6 IMAGE PROCESSING AND ANALYSIS

The fluorescent images from the Zeiss Axiovert and Nikon microscopes were converted to 8-bit tiff files and analyzed using the NeuronJ plug-in for the ImageJ software. The purpose of this analysis was to characterize and identify whether any order was imposed on neurons developing in the presence of the NTbs, compared to the SiO$_2$ or pSiN$_x$ substrates. Using NeuronJ, the MAP2 images were loaded and the dendrites of each cell were manually traced (Figure 3.3).

![Figure 3.3: Example of NeuronJ Tracings: (a) NTb-PEFC (4 DIV) MAP2 image prior to NeuronJ reconstruction (10x, scale = 50 μm); (b) NTb-PEFC (4 DIV) MAP2 image post NeuronJ reconstruction (10x, scale = 50 μm).](image)

For each NTb array, a tracing was performed on a brightfield image to establish the reference angle, $\theta_{ref}$, provided by the NTbs. Then, the (x,y) coordinates of the origin and terminal point of each process were exported and saved into a distinct data file.
corresponding to each experimental condition. These files were called in a MATLAB script and converted to polar coordinates, of the form \((\theta_i, \rho_i)\), where:

\[
\theta_i = \tan^{-1} \frac{y_i}{x_i} \quad (Equation \ 1)
\]

\[
\rho_i = \sqrt{x_i^2 + y_i^2} \quad (Equation \ 2)
\]

For the NTb data, the angle of each process was normalized to the reference angle with the following equation:

\[
\theta_{\text{norm}} = \theta_i - \theta_{\text{ref}} \quad (Equation \ 3)
\]

Finally, the coordinates \((\theta_{\text{norm}}, \rho_i)\) were converted back to Cartesian coordinates and plotted with the compass\((x,y)\) function and the plots were generated in MATLAB, these plots are shown in Chapter 4.

3.7 STATISTICAL ANALYSIS

The results for the number of branches per cell are presented as the mean and standard error. The statistical analysis performed on these data was a one-way ANOVA.

For the data from the compass plots in MATLAB, the angle data were extracted and an angular histogram, or rose diagram, of the collection of angles for each experimental condition was generated, with the data divided into 36 bins, each spanning 10°. The variance of the histograms is presented. Additionally, the means for the angles and magnitudes were calculated and the mean and standard deviation are presented. A one-way ANOVA was performed on these data.

Results were judged significant if the p-value was < 0.05.
4.1 HIPPOCAMPAL NEURON GROWTH ON NEUROTUBE ARRAYS

Within this section, the most interesting images from each of the experimental conditions are shown and discussed. However, the chosen images are representative of the other images within the data set of interest. For comparison, a representative image of the pSiNₓ control and SiO₂ control can be found in Figure 4.5 at the end of Section 4.1.

Figure 4.1: Hippocampal neurons (3 DIV) cultured on NTb-FE substrate extend dendrites through adjacent NTbs. (a) Brightfield image of the NTb array for orientation in (b). The NTbs, 50 µm long structures aligned horizontally in the image, are identifiable (10x, scale = 50µm); (b) P2 hippocampal neurons at a density of 600 cells/mm², cultured for 3 days on the fully etched NTb substrate, stained with MAP2 (10x, scale = 50 µm); (c) Depicts the area highlighted by the orange box in (b), rotated 120° counterclockwise (CCW). The orange arrows indicate the location of two NTbs (40x, scale = 50 µm); (d) SEM image of neuron cluster highlighted by the orange box in (b), rotated 120° CCW. The orange arrows indicate the same NTbs identified in (c) (1195x, scale = 50 µm).
In Figure 4.1, a collection of images from the 3 DIV culture on the fully-etched NTb substrate is shown. The brightfield image in Figure 4.1a serves to orient the viewer to the NTb array. This culture was seeded at a relatively high density to ensure the adherence of neurons by flooding the substrate with a higher density than used for the low-density cultures.

Of particular interest in this set of images is the group of neurons on the left side of the image in Figure 4.1b (highlighted by the orange box). One of these neurons has two processes that grew towards two NTbs and turned into the tubes, growing through the length of the tube. Since the NTbs are optically transparent, each process can be observed within the NTb due to the MAP2 label. SEM was performed to further investigate the interaction between the two dendrites and the NTbs, and verified the connection and entry of each process into its respective NTb (Figure 4.1d).

The images in Figure 4.2 are from the partially etched, fully collapsed NTb substrate. This set of images is of particular interest because it illustrates how the dendritic processes will actually turn to make a connection with the NTb (Figure 4.2b).

In Figure 4.3 a collection of SEM images from the NTb-PEFC substrate are shown. On this substrate, part of the sacrificial layer remained (partial etch) such that the NTbs did not roll. The SiOx anchor is visible as the thin grey outline on the right side of the NTbs in Figure 4.3a,b. In the SEM images, it is clear that some NTbs have dog-eared corners, while others exhibit a half turn, or partial roll. These images provide additional insight on the neuron-NTb interactions. For instance, in Figure 4.3a the neuron has a process connected to the underside of the NTb, what would effectively be the inner membrane, had it rolled. It is possible that the neuron’s process actually adhered to the SiNₓ membrane while it was still unrolled and exerted enough tension to pull the membrane over into the dog-eared position.

In Figure 4.3b, the soma appears to be attached to the right NTb while its lower process reaches across the gap between NTbs to connect to the left NTb membrane. Interestingly, both processes and the soma are attached to the outer side of the membrane, which is semi-rolled. This behavior and that of the cells and processes in Figure 4.3c, which appear to be avoiding contact with the SiOx anchor and preferentially adhering to the SiNₓ membrane, suggest that the neurons may prefer the SiNₓ to the SiOx, when given a choice. This theory must be tested empirically.
Networks among the neurons appear organized along the NTbs (Figure 4.3d,e). In Figure 4.3d, it appears that the somas are missing; however some processes are still attached, forming a type of bridge between the NTbs. In Figure 4.3e, several neurons are connected in a roughly shaped circle, with some processes directed through the inner membrane of a NTb.

Figure 4.2: Dendrites from hippocampal neurons (4 DIV) cultured on NTb-PEFC substrate will turn to grow through a NTb. (a) P2 hippocampal neurons seeded at a density of 200 cells/mm², cultured for 4 days on the partially etched, fully collapsed NTb substrate, stained with MAP2 (10x, scale = 50 µm); (b) Depicts the area highlighted by the solid orange box in (a). The orange arrows indicate the location of a NTb (40x, scale = 50 µm); (c) Depicts the area highlighted by the dashed orange box in (a). The orange arrows indicate the location of NTbs (40x, scale = 50 µm); (d) Brightfield image of the NTb array for orientation purposes. The NTbs, 50 µm long structures aligned horizontally in the image, are identifiable. The orange arrows highlight the NTbs identified in (b) and (c) (10x, scale = 50µm).
Figure 4.3: Interactions of hippocampal neurons (4 DIV) and their processes with NTb-PEFC substrate, SEM. (a) A neuron between two NTbs with processes on the inner membrane of each NTb (2612x, scale = 20 µm); (b) A neuron suspended between two NTbs with processes anchored to the outside edge of each NTb (2612x, scale = 20 µm); (c) A cluster of neurons send processes to multiple NTbs (1306x, scale = 50 µm); (d) Processes grow along the NTbs (1500x, scale = 20µm); (e) A network of neurons aligned to form the rough shape of a circle as processes grow through neighboring NTbs (653x, scale = 100 µm).
The images in Figure 4.4 are from the ghelical NTb substrate and show one particular helical NTb-neuronal association of interest. This NTb has four cells growing along its length, one collection of three neurons (shown in Figure 4.4b) and a single neuron near the end of the NTb (Figure 4.4c). This same NTb was found on the SEM.
and the cluster of three neurons is pictured in Figure 4.4d. The somas are aligned on either side of the NTb, and the processes from the cells tend to follow the edge provided by the length of the NTb.

Figure 4.5: Hippocampal neurons (4 DIV) on pSiN, and SiO2 controls show random process extension. (a) P2 hippocampal neurons cultured for 4 days on the pSiN control, stained with MAP2 (10x, scale = 50µm); (b) P2 hippocampal neurons seeded at a density of 150 cells/mm², cultured for 4 days on the SiO2 control, stained with MAP2 (10x, scale = 50 µm).

Although is it apparent from the images in Figures 4.1-4.4 that the neuronal processes will either align with the NTb and grow along its exterior edge, or find their way to the opening of the NTb and extend through the NTb, this is not the case for every cell. On average, the soma must be within 83 µm for its process to touch a NTb, and within 77 µm in order for the process to align with the NTb (Table 4.1). On the NTb substrate, for every cell that connects with a NTb, only about 56.6% of the processes that come into contact with a NTb align along either the exterior edge, or inner membrane. Overall, the there are more processes that do not align; this lack of interaction is further examined and quantified in Section 4.3.

<table>
<thead>
<tr>
<th></th>
<th>d_{min} (µm)</th>
<th>d_{max} (µm)</th>
<th>d_{avg} (µm)</th>
<th>% Aligned</th>
</tr>
</thead>
<tbody>
<tr>
<td>All processes touching a NTb</td>
<td>1.538 ± 0.639</td>
<td>83.458 ± 15.941</td>
<td>19.575 ± 3.333</td>
<td>56.619% ± 11.589%</td>
</tr>
<tr>
<td>Aligned processes</td>
<td>1.543 ± 0.636</td>
<td>76.880 ± 13.718</td>
<td>14.754 ± 3.216</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Distance between NTb and soma with established connection. The minimum (d_{min}), maximum (d_{max}), and average (d_{avg}) distance between a soma and a NTb upon which at least one process from that cell is either touching the NTb, or aligns with it (± standard deviation).
4.2 COMPARISON OF NEURON GROWTH AMONG NEUROTUBES, SiO₂, AND SiNx

An important question to answer is whether the NTb substrate has a positive or negative affect on the branching and development of the neurons. The materials (pSiNx and SiO₂) are both biocompatible, so no adverse effects were expected. In fact, either similar, or enhanced branching was expected on the NTb substrate, compared to the pSiNx, and SiO₂, respectively, since SiNx is a softer substrate than glass, and neurons have an affinity for soft substrates.

In order to quantify any difference among the three substrates: NTb, pSiNx control, and SiO₂ control, the number of processes ($P_{\text{total}}$) for each dish was calculated using NeuronJ and summed for each condition, and the number of cells ($C$) for each dish, with reconstructed processes, was counted manually and summed for each condition. The average number of processes per cell for each condition was calculated as follows:

$$P_{\text{average}} = \frac{P_{\text{total}}}{C} \quad (\text{Equation 4})$$

The ANOVA returned a $p > 0.05$ indicating no significance between any combination of the three substrates. The results, with standard error bars, are shown in Figure 4.6.

![Processes per Cell](image)

**Figure 4.6: ANOVA Results: Processes per cell.**

$N_{\text{SiO₂}} = 3$; $N_{\text{pSiNx}} = 3$; $N_{\text{NTb}} = 5$; Number of cells per condition: SiO₂ = 117; pSiNx = 231; NTbs = 739; $p$-value $> 0.05$
This result is promising because it indicates: (1) there is no significant difference in the number of processes on any of the three substrates, which implies (2) the neurons grow as well on the NTb substrates as they do on either pSiNₓ or SiO₂ (which is a standard substrate for neuron culture). Had there been a diminished process count on the NTb substrates, this would have indicated decreased health of the neurons, but the absence of significant variance in the number of processes that arise on the NTb substrate compared to the two controls speaks to the health of the neurons on this substrate. This result confirms the biocompatibility of the substrate and justifies the use of this platform for studies with neurons.

4.3 CHARACTERIZING ORIENTATION CUES PROVIDED BY NEUROTUBES

Given what is known about topographical cues and neuron growth, there was some concern that the NTb substrate provides a significant guidance cue to the neurons. The extent of this guidance must be characterized before the NTbs are functionalized with electrodes so that electromagnetic (EM) fields can be applied to the system (non-powered vs. powered). If there is a significant orientation factor provided by the NTbs, this must be mapped to provide a baseline for future experiments with the powered NTbs. This will make it possible to separate guidance as a factor of the EM fields, as opposed to the topography of the NTbs.

In order to assess the presence or absence of uniformity on the NTb substrates, polar plots for each condition were constructed, as described in Chapter 3.6. These plots are shown in Figures 4.7 and 4.8. Each of these plots contains a collection of vectors corresponding to the direction of each process on the substrate. The vectors are plotted on a unit circle to facilitate visualization of deviation of each element from the origin. In the case of the NTbs, all the vectors were normalized to the orientation of the array. For the controls, there was no normalization since the control substrates lacked directional cues.

Analysis of the polar plots show, that there is no clear uniformity of process orientation in the NTb substrates. Similar to the control plots, the vectors have a wide distribution from 0° to 360°.
Figure 4.7: Polar plots of neurite orientation on various NTb substrates. (a) NTb-FE (3 DIV); (b) NT-FE (4 DIV); (c) NTb-PEFC (4 DIV); (d) NTb-helical (4 DIV); (e) NTb-ghelical (4 DIV).
To further visualize the random distribution of the processes, a rose diagram was generated for each data set contained in the plots in Figures 4.7 and 4.8. The angle data for each condition were divided into 36 bins, each spanning 10°. Each bin becomes a

Figure 4.8: Polar plots of neurite orientation on pSiNₓ and SiO₂ controls. (a) pSiNₓ (3 DIV); (b) pSiNₓ 1 (4 DIV); (c) pSiNₓ 2 (4 DIV); (d) SiO₂ (3 DIV); (e) SiO₂ 1 (4 DIV); (f) SiO₂ 2 (4 DIV).
wedge within the rose diagram, and the wedges illustrate the frequency of processes oriented within a 10° span (e.g. there are 15 processes oriented within 0°-10° from the origin on the PEFC NTb substrate, Figure 4.9c). The rose diagrams are shown in Figures 4.9 and 4.10.

Figure 4.9: Rose plot/angular histogram of angle, $\theta_{\text{norm}}$, for NTb substrates. The data are divided among 36 bins, each spanning 10°. $\theta_{\text{norm}}$ represents the angle of each process from the origin. The length of each wedge indicates the number of processes that are contained within a span of 10°, e.g. 0°-10° or 260°-270°. (a) NTb-FE (3 DIV); (b) NT-FE (4 DIV); (c) NTb-PEFC (4 DIV); (d) NTb-helical (4 DIV); (e) NTb-ghelical (4 DIV).
Inspection of the rose diagrams demonstrates a wide distribution of the frequency of angles in each condition. If there were an overarching order imposed by

Figure 4.10: Rose plot/angular histogram of angle, $\theta_{norm}$, for pSiN$_x$ and SiO$_2$ controls. The data are divided among 36 bins, each spanning 10°. $\theta_{norm}$ represents the angle of each process from the origin. The length of each wedge indicates the number of processes that are contained within a span of 10°, e.g., 0°-10° or 260°-270°. (a) pSiN$_x$ (3 DIV); (b) pSiN$_x$ 1 (4 DIV); (c) pSiN$_x$ 2 (4 DIV); (d) SiO$_2$ (3 DIV); (e) SiO$_2$ 1 (4 DIV); (f) SiO$_2$ 2 (4 DIV).

Inspection of the rose diagrams demonstrates a wide distribution of the frequency of angles in each condition. If there were an overarching order imposed by
any condition, an obvious direction would reveal itself in the diagram, with the majority of values (each representing a single process) contained in the same bin. Instead, for each condition: NTb, pSiNx, SiO₂ and the replicates contained therein, the data are distributed widely across the bins. Although Figure 4.10e appears slightly ordered along 150° and 330°, these results are from a single SiO₂ condition so they must be considered along with the results in Figure 4.10d,f; when combined, there is no significant difference in the variance among the three conditions (NTb, pSiNx, SiO₂). This observation is confirmed by the ANOVA of the variance (Figure 4.11).

The variance describes how far away each value is from the mean of the data set. If the magnitude of the variance is large, there is a wide distribution of the values within the range, whereas a small magnitude indicates a tight distribution. In this case, the variance for each condition is large: greater than 300 deg², and there is no statistically significant difference among the three groups, which indicates that no one group is any more biased towards a specific angle than any other.

![Variance of Theta](image)

**Figure 4.11: ANOVA Results: Variance of theta.** The variance for each condition (SiO₂, pSiNx, and NTb) was calculated and a one-way ANOVA was performed. N₃₅O₂ = 3; N₃₃Nₓ = 3; N₅NTb = 5; Number of angles per condition: SiO₂ = 448; pSiNx = 695; NTbs = 1,787; p-value > 0.05

These results, taken together with those discussed in Section 4.1, indicate that the NTb substrate does not provide a strong signal for uniform directional growth of neuron processes by virtue of its topography alone. In light of this fact, as this project moves forward and the NTbs are powered, changes observed in future experiments, including
polarization of the neurons and their processes, can be attributed to the introduction of electric and magnetic fields since the surface cues alone are not enough to significantly change the outcome compared to the controls.
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 INTERACTION BETWEEN NEURONS AND NEUROTUBES

Within this thesis a novel NTb platform was introduced, and the extent to which the NTbs influence the orientation of hippocampal neurites was explored. The rationale for this preliminary study, prior to functionalizing the NTbs with electrodes, was driven by the knowledge that topographic cues influence the growth of neurites. Based on the experiments conducted with globally applied EFs, it is expected that the neurites will grow in the direction of the field, however, in the global studies, there were not substrate cues. In order to confidently interpret the results of NTb experiments with applied EFs, it was necessary to first determine how the processes behave with the non-powered NTbs.

The results are promising: the NTbs do not have an overwhelming effect on the directionality of the processes. In fact, there was no significant difference among the NTbs and the two controls with respect to the alignment of the processes in a single direction: all conditions showed a wide variance against their respective means, meaning that no one group was any more biased towards a specific direction than any other group. Although some processes did align with NTbs, either along an edge, or through the inner diameter of the NTb, only a subset of the fraction of processes that connected with a NTb showed alignment (Chapter 4.1: Table 4.1). The instances of alignment frequently occurred when the tip of a process found either the opening of a NTb, or one edge of the NTb. However, some processes were found to align along only half of the length of the NTb, and then deviated from the cue provided by the NTb. Furthermore, when considering the subset of process that align (329) from the total number of processes (2,062) on each NTb substrate, the average likelihood of alignment drops to only 17.027% ± 10.399% (data not shown). Additionally, quantification of the distance between the somas and NTbs indicates that a soma must be within about 77 µm in order for its process to align with the NTb. Since the seeding of neurons is random (500 µL of cell suspension with a density of 150-200 cells/mm² is deposited on the
device, and the cells adhere without direction), in the absence of electric signals, the incidence of alignment should remain low, and the random growth will prevail.

5.2 LOOKING FORWARD: POWERED NEUROTUBES

To date, the majority of experiments exploring the effects of applied electric fields and stimuli on neurons have focused on axonal development or complex neural networks. The results presented in Chapter 4 are concerned with the orienting effects of the NTbs on the dendrites of the hippocampal neurons; however, in addition to quantifying the response of the dendrites, the larger goal of this work is to delve down to an even smaller scale and illuminate the impact electrical stimulation has on dendrites by way of the dendritic filopodia, through the study of these interactions at a single-cell level. As mentioned previously, by working with low-density cultures on the NTb platform, it will be possible to selectively stimulate individual neurons and elucidate how the behavior of the filopodia impacts the overall organization of the dendritic architecture. This will provide a critical piece to the puzzle of neural network formation. Since dendritic filopodia are often the precursors to stabilized spines, the outcome of this research stands to offer further insight into the dynamics of dendritic spine stabilization, a critical component of memory formation and recall.

Moving forward, the next steps will be to: fabricate the NTbs integrated with electrodes; characterize the EF lines on a device without cells; culture the neurons on the powered NTbs with varied electrical parameters; investigate how the EFs influence the growth of the processes; record from the processes within the NTbs to explore inherent fields, and compare responses in the absence of EF stimulation with those in the presence of EF stimulation.

5.3 DISEASE MODELS AND CLINICAL RELEVANCE

Aside from adding to the foundation of the basic science of hippocampal dendritic development, this project has strong applications to several disease models. From a clinical perspective, a deeper knowledge of filopodial dynamics and spine formation can impact our understanding of developmental disorders like autism and Fragile X Syndrome (FXS), and may facilitate new approaches in treatment and management of neurological disorders such as schizophrenia.
Autism spectrum disorder (ASD) is a developmental disorder that manifests as impaired social interaction, with a range of severity. The genetic basis for autism is now fairly widely accepted since the probability the sibling of a child with autism will also be autistic is 2-3%, which is significantly higher than the incidence in the general population (0.005%-0.1%) [72]. In patients with autism, MRI imaging revealed structural differences in elements of the limbic system such as the hippocampus, including reduced density of the tissue, diminished cell size, and reduced complexity in the dendritic arbor, when compared to healthy subjects [20]. Moreover, a specific gene has recently been implicated in autism: diaphanous homolog 3 (DIAPH3), which plays a role in cell migration, axon guidance, and neurite development and growth [73]. The loss of this gene, through a deletion on one allele and a point mutation on the other, leads to reduction of filopodia; this result was experimentally determined in mice with the DIAPH3 construct, compared to wild-type (WT) mice. The expectation is that DIAPH3 is involved in the actin dynamics of neuronal filopodia [73].

FXS is another developmental disorder that affects filopodial dynamics. People with FXS do not produce the fragile X mental retardation protein (FMRP) due to the CCG residues repeated hundreds or thousands of times on the fragile X mental retardation 1 (FMR1) gene on the X chromosome (instead of 5-50 times in unaffected individuals) [72]. Hippocampal neurons from FMRP-deficient mice were compared to those from WT mice following culture for 3 weeks. The dendrites of neurons in a mouse with a genetic deletion for FMRP (a gene knock-out animal, KO) were shorter with fewer spines, and overall, there were fewer functional synapses in the KO cultures [74]. In order for spine formation to occur, FMRP must not only be expressed, but it must co-localize with dendritic mRNA. When the transport of the mRNA was disrupted in WT cultures that exhibited increased filopodial extensions, which were expected to precede spines, the formation of spines was disrupted. This disruption resulted in a significant increase in the length and density of the filopodia, but they were unable to mature into spines; a phenomenon similar to what occurs in FXS [75].

In addition to developmental disorders, there are many neurological disorders that involve the disruption of a cellular process or structural organization. The gene DISC-1 (disrupted-in-schizophrenia-1) is predominately expressed in the hippocampus, and variation in the alleles can lead to changes in the level of engagement
of the hippocampus during cognitive tasks (determined through MRI) [76]. DISC-1 has been shown to localize completely in the perinuclear punctate structures that extend into the neuron processes. Critically, it co-localizes with the centrosomal complex through the help of MIPT3 and taxol (a stabilizer). In cells with a truncated DISC1, the location becomes more diffuse and more abundant in the neurites, although it still co-localizes with the centrosomes with MIPT3 and taxol. The suspicion is that the truncation of DISC-1 predisposes a person for schizophrenia because it disrupts intracellular transport, and subsequently the formation of neuronal architecture [77].

Clearly all three of these disorders are tied to filopodial dynamics. Following the quantification of the normal electrical signals of dendrites and filopodia, and the characterization of how they can be manipulated with EFs, it is logical to move to a disease model, such as FMRP KO mice. The hippocampal neurons from these mice can be isolated and cultured in the same manner as the WT. Their inherent electrical signals will be recorded, and their growth patterns quantified to establish the baseline. Then, the KO neurons can be cultured with electrical stimulation, with the goal to modulate the applied stimuli until the development of the KO neurons mimics that of the WT neurons. The desired outcome is effectively a form of electrical therapy tuned to the subcellular dynamics in order to correct the developmental deficiencies.
REFERENCES


