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Studies on Neuron Responses to Simultaneous and Competing Extracellular Cues

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Studies on Neuron Responses to Simultaneous and Competing Extracellular Cues

by

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Dedication

This dissertation is dedicated to my parents, Alberto and Beatriz, my sisters, Patricia, Amparo, Sonia and Adriana, and my fiancé, Luis Eduardo.

Esta disertacion esta dedicada a mis padres, Alberto y Beatriz, mis hermanas, Patricia, Amparo, Sonia y Adriana, y a mi prometido, Luis Eduardo.

Sin su apoyo constante y su infinita confianza en mi, nunca lo hubiera logrado.
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The understanding of cell responses to extracellular signals is a field of major importance. In particular, cell interactions with designed surfaces are fundamental for creating successful interfaces between biomaterials and biological entities. Bioactive materials that promote specific behaviors such as cell proliferation, migration or differentiation are highly relevant for tissue engineering strategies. In particular, neural engineering applications including nerve conduits for nerve regeneration, neural prosthetics and artificial neural networks, would be highly benefited from studies on artificial substrates that modulate neuronal behavior.

Extracellular stimuli influence critical phases in neuronal development such as neuron polarization (i.e., axon formation) and axon growth. There are numerous stimuli that have been studied for influencing neuron responses. Some of these stimuli are substrate topography, growth factors, extracellular matrix components, electrical activity and the presence of support cells. Although all
these stimuli induce responses in neurons independently, in this investigation we focused on cell behavior when simultaneous cues were presented to the cell. This included both combinatorial and competitive cues.

We studied the novel combination of chemical and physical stimuli by immobilizing nerve growth factor (NGF) on topographical features. We found that topography (i.e., physical stimulus) highly influences axon formation, whereas axon extension is controlled by a synergy of immobilized NGF and topography. We also investigated the combination of electrical and chemical stimulation. In that case, NGF was immobilized on polypyrrole, an electrically-conducting polymer, finding an enhanced effect on neurite extension. Finally, simultaneous but spatially independent stimuli (i.e., competitive stimuli) were investigated for influencing direction of polarization. We found that physical cues were preferred over chemical cues for axon formation.

Our results have contributed to further knowledge regarding the modification of artificial substrates that better control neuronal responses. This knowledge can be applied in the design of materials for nerve regeneration strategies, the modification of electrodes that stimulate neurons in prosthetic devices, and for the control of neuronal mapping and connectivity in neural nets.
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Chapter 1
Introduction

The creation of biomimetic interfaces has evolved as a field of great importance for biomedical applications. A synthetic material can be rendered “biomimetic” by modifying it with “natural” elements that induce very specific cellular behaviors such as survival, differentiation, migration, proliferation, attachment or neurite extension. Interactive surfaces that modulate cell behavior are essential for multiple applications where control of cell behavior is required. In regenerative medicine, implantable scaffolds and devices are designed with the purpose of aiding regeneration of tissue in the body. In this application, material surface properties are important to induce cell recognition and tissue formation. In particular to neural tissue engineering, optimal interfacing between artificial materials and neurons is critical for neural prostheses, nerve regeneration strategies and the creation of artificial networks.

1.1 Cell Interactions with Engineered Materials

Tissue engineering is an emerging field that combines concepts and techniques from engineering and life sciences for the creation of artificial constructs that enable tissue regeneration or replacement [1]. In tissue engineering, biomimetic or bioactive materials are key factors for regulating and controlling cell behavior to ultimately promote regeneration [2,8]. (Figure 1.1).

Several strategies have been evaluated for the creation of bioactive materials. Incorporation of extracellular matrix components (ECM) molecules such as laminin, collagen and fibronectin, or peptides such as RGD, YIGSR and IKVAV, has been recognized as a valuable tool for promoting cell adhesion to biomaterial surfaces [3,4]. Similarly, incorporation of growth factors is critical, as these
proteins are potent modulators of many cellular responses including proliferation, migration and differentiation [2]. With the purpose of presenting these molecules to cells, techniques such as protein adsorption, protein and peptide immobilization, and protein encapsulation in three-dimensional (3-D) scaffolds have been explored [4] (Figure 1.2). In addition, the importance of material physical architecture has been recognized, promoting the creation of 3-D materials and surfaces with micro and nanotopographies [5,6]. Alteration of surface topography has profound effects on cells, including changes in adhesion, orientation, motility, cytoskeleton conformation, tyrosine kinases activation and gene expression [7].

Figure 1.1. Cell communication with biomimetic and bioactive materials. Presence of ECM components, growth factors, and cell-cell interactions determine the fate of cells on biomaterials in tissue engineering strategies. Figure adapted from [8].
1.2 Neural Tissue Engineering

Neural tissue engineering applies strategies for creating biomimetic materials that are interfaced with neurons. In this field, strategies for regeneration of nerves after injury have been extensively explored [9-11]. One particular therapeutic strategy is the implantation of nerve guidance channels in the gaps where nerves have been severed because of a tumor or trauma (Figure 1.3). The ultimate goal of these nerve conduits is to provide support and the appropriate environment and stimulation for axons to regrow through the conduits and reconnect with target cells to eventually regain function.

The design of nerve guidance channels is fundamental to provide the correct combination of cues and signals to guarantee that axons enter the conduit, grow with a specific direction, and finally exit the conduit. For this design, multiple stimuli and material properties have been explored to enhance axon growth and orientation. For example, some of the important properties for nerve conduits are biocompatibility, biodegradability, and the incorporation of physical
guidance cues, electrical stimulation, growth factors, ECM components, and support cells such as Schwann cells [9].

![Nerve Injury](image)

**Figure 1.3.** Nerve guidance channels. Nerve conduits are designed to provide support for regenerating axons and stimulating signals such as growth factors, physical guidance cues and electrical stimulation. Figure adapted from [10].

*Neural prosthetics* is another important application in neural engineering. Many traumas and diseases affecting nerves generate permanent loss of a particular function in the body or paralysis because of the inability to communicate and transport signals to muscles. A neural prosthesis is a device that assists or replaces a lost function by stimulating and receiving signals that cannot be perceived by the damaged organ or nerve. The main function of the prosthesis is to provide an electronic instrumentation that acquires the signals from the exterior or neurons and conveys sensory information or motor actions to other parts of the nervous system [12]. The interface between the implanted probe and the surrounding tissue is critical for obtaining optimal integration of signals. Additionally, biocompatibility, mechanical properties, durability and size of the neural probe are important parameters to consider in the design of the device [13]. Good interfacing can be achieved by modifying electrode surfaces
for presenting cues that stimulate growth and recognition by neurons, including the release of neurotrophic factors such as nerve growth factor (NGF) [14], one of the molecules investigated in this dissertation.

Different types of neural prostheses have been designed for several applications. One of the first successful implants was a cochlear prosthesis, which consists of an external microphone that collects sounds that are subsequently transmitted to an electrode array that electrically stimulates auditory neurons (Figure 1.4A) [13,15]. Retinal implants also offer a promising solution for patients with retinal diseases. In this case, a photodiode array receives visual information and transmits it to a stimulating array of electrodes interfaced with the optical nerve (Figure 1.4B) [12,13].

Figure 1.4. Implantable neural prostheses. A) Cochlear implant; B) Retinal implant. When the optic nerve is damaged, an epicortical implant can be used instead to directly stimulate the brain. Figures adapted from [13].

Finally, the creation of artificial neural networks also highly depends on interactions between neurons and artificial materials. In this application, a small number of neurons are patterned on an array of electrodes that can either stimulate or record from individual neurons (Figure 1.5). Neurons extend axons and create synapses with neighboring cells, which allows the study of neuronal activity and communication in the nervous system. Fromherz has highlighted the
importance of controlling the patterning ("mapping") and connectivity of neurons to produce successful neural nets [16,17]. In particular, controlling axonal outgrowth orientation and synapses is critical for the understanding and reproducibility of the network characteristics. With this purpose, patterning of adhesive molecules and topographical features have been investigated to direct polarization of neurons and axon orientation in neural nets [18-20]. However, more studies on successful modification of material surfaces that control neuronal behavior are required.

Figure 1.5. Artificial neural networks. A small number of neurons are patterned on an array of electrodes. Neurons extend axons and create synapses, which allows the study of neuronal communication in the nervous system. Figure modified from [17].

1.3 Overview of Dissertation

The preceding sections emphasized the dramatic role of material design for effective interfacing with neurons in applications such as nerve conduits, neural prosthetics and neural networks. In light of this need, this dissertation has focused on the modification of material surfaces to be interfaced with neurons. In particular, modifications with immobilized growth factors and topographical
features have been investigated to influence neuronal polarization (i.e., axon formation) and axonal outgrowth.

The focus of this dissertation was to analyze first, neuron responses to novel combinations of extracellular cues, and secondly, neuron responses to competitive signals. All cues were designed to be presented as properties of a biomaterial for potential biomedical applications. Three types of stimuli were investigated in this project: 1) chemical stimulation by surface-immobilized growth factors, 2) physical stimulation by substrate microtopography, and 3) electrical stimulation by conducting substrates.

Chapter 2 overviews the effect of extracellular cues in neuronal behavior, including neuron polarization and axon guidance. A broad description of the most important environmental stimuli is provided, including 1) chemical stimulation by neurotrophins, netrins, semaphorins, slits and extracellular matrix (ECM); 2) physical stimulation by topography, tension and substrate stiffness; 3) interaction with glial cells and 4) electrical stimulation.

Chapter 3 describes in detail the background literature for each one of the studied extracellular cues in this investigation. The model growth factor used in this investigation was nerve growth factor (NGF), which was immobilized on the surface of materials. The characteristics and immobilization techniques for this protein are discussed in this chapter. Secondly, surface topography provided by microchannels is explained, including the different microfabrication methods. Finally, regarding electrical stimulation, the conducting polymer polypyrrole is described, including polymerization and modification techniques.

Chapter 4 explains the fabrication of microchannels using soft lithography methods. A common elastomeric material called poly(dimethyl siloxane) (PDMS)
was chosen as the substrate for incorporating microchannels of different dimensions. For this fabrication, masters were created on silicon wafers by using electron beam (e-beam) lithography, followed by chromium lift-off and reactive ion etching (RIE). These masters served as molds for replica molding of PDMS. Characterization of the masters and final PDMS substrates was performed using light microscopy (transmitted and reflectance), scanning electron microscope (SEM) and atomic force microscopy (AFM).

Chapter 5 discusses the first combination of extracellular cues investigated in this dissertation. Microchannels on PDMS were chemically modified with immobilized NGF to render the surface bioactive. By doing this, a combination of contact guidance and chemical guidance with growth factors was achieved. Embryonic hippocampal cells were used to investigate the effects of these modified surfaces on neural behavior. In particular, neuron polarization and axon elongation were analyzed. Results demonstrated that topographical stimuli dominate polarization, whereas the combination of topography plus NGF dominate overall axon length.

Chapter 6 describes the second combinatorial strategy. In this case, polypyrrole surface was coated with immobilized NGF. This modification provided the combination of electrical stimulation plus chemical stimulation with growth factors. PC12 cells were used to analyze neurite extension on polypyrrole-NGF, finding an increase in neurite length when current was passed through the material and NGF was tethered to the surface.

Chapter 7 explains the first part of the strategy to combine microchannels, immobilized NGF and electrical stimulation in a single material. For this approach, polypyrrole microchannels were fabricated on indium tin oxide (ITO) slides using e-beam lithography and electropolymerization. These features can
be potentially modified with immobilized NGF for obtaining the triple combination in future studies. In this chapter, polypyrrole microchannel fabrication and the effect of these topographical features on polarization and axon length of hippocampal cells are described.

In Chapter 8, analysis of competitive responses between microchannels and immobilized NGF is described. In this particular study, the two stimuli were simultaneous but not superimposed as in the studies described in Chapter 5. On the contrary, the two cues were independently presented in a parallel fashion. Hippocampal neurons were used to analyze competitive responses by determining the side of polarization. Results showed that physical guidance cues were preferred over chemical cues for polarization.

Finally, Chapter 9 summarizes the results of the project and future directions, including the triple combination of electrical stimulation with growth factor and topography, and other possible competitive responses in axon guidance.

1.4 References


Chapter 2
Extracellular Stimuli and Cellular Responses

Introduction

The fields of tissue engineering and biomimetic materials have applied numerous approaches for the modification of artificial materials to incorporate extracellular cues that stimulate cells. These stimuli can be bioactive molecules (e.g., extracellular matrix (ECM) components, growth factors), physical signals (e.g., topography, roughness, substrate stiffness), cellular signals (e.g., support cells) and electrical stimuli. This chapter describes first, the importance of extracellular cues in determining neuron behavior, giving special emphasis to polarization and axon guidance. Secondly, a broad description of the most important environmental cues is provided. This overview includes multiple stimuli that were not directly investigated in this dissertation, but could be used for future applications.

2.1 Influence of Extracellular Cues on Neuronal Behavior

2.1.1 Growth Cones and Rho GTPases

Neurons are the key functional cells that form the central and peripheral nervous systems. A neuron consists of a cell body, dendrites and an axon. The axon contains three types of cytoskeleton structures: microtubules, actin filaments and intermediate filaments [1]. The growth of an axon strongly depends on actin polymerization, which forms microfilaments that organize into higher-order structures (lamellipodia, filopodia and stress fibers) to create tension in the cellular membrane to form a specific shape. The growth cone, an actin-rich specialized motile structure, is located at the tip of the axon (Figure 2.1). It is
formed by lamellipodia (web-like veils) and filopodia (finger-like protrusions) that extend and sense the surrounding environment [2].

Figure 2.1. Schematic of a growth cone. Figure adapted from [2].

The growth cone is highly sensitive to extracellular cues, and the integration of attractive and repellent signals determines the extension and steering of the axon. Actin polymerization and rearrangement is controlled by GTPases of the Rho family (Cdc42, Rac and Rho) [2,3]. These proteins are molecular switches that when activated in response to external cues, trigger actin polymerization and bundling [1]. In general, Rac and Cdc42 activation promotes actin polymerization, whereas Rho activation causes retraction. Therefore, attractants promote actin polymerization by activating Rac and Cdc42 and inhibiting Rho, whereas repellents inhibit Rac and Cdc42 and activate Rho.

Rho GTPases are active when GTP is bound to the molecule, and inactive when GDP is bound [1]. When active, Rho GTPases interact with downstream proteins that initiate actin polymerization. These GTPases are controlled upstream by other proteins. In particular, guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP activating the GTPases, whereas GTPase-activating proteins (GAPs) facilitate the opposite exchange, inactivating these. GEFs and GAPs are activated by multiple intracellular pathways initiated by extracellular molecules [3].
2.1.2 Neuron Behavior

2.1.2.1 General Remarks: Neuronal Development

Neuronal behavior is profoundly influenced by extracellular signaling in many phases (Figure 2.2). In embryological neural development, new-born neurons derived from epithelial cells at the neural tube migrate to specific locations before extending an axon and dendrites [4]. The first step in this migration process is the extension of a leading edge consisting of filopodia and lamellipodia that explore the external environment and integrate guiding signals, followed by movement of the nucleus and retraction of the trailing side [5].

![Figure 2.2. Neuronal behavior influenced by extracellular cues. Migration, polarization and axon guidance are controlled by interactions with extracellular cues such as glial cells, ECM components and growth factors. Adapted from [5] and [14].](image)

After neurons migrate, they follow a process of polarization, in which cells extend neurites in all directions, but only one of these becomes an axon while the others become dendrites [6]. This polarization is crucial for almost every neuronal
function. The process has been demonstrated to be modulated by extracellular cues [7].

Finally, at a later stage after the axon is clearly established, the combination of signals at the growth cone determines the specific pathway by which axons travel toward their target cells and establish connectivity [2,8,9]. In addition, the same type of situation arises in peripheral nerve injuries when regenerating axons extend actin-rich growth cones that actively migrate along the substratum to finally reconnect with the target cells [3].

It is well known that environmental cue integration occurs at the individual growth cone in the case of axon steering [2,3]. However, integration of extracellular signals throughout the complete cell body also plays a role [10], in particular for neuron polarization [12,13]. We have analyzed this important concept as described in Chapter 8.

2.1.2.2 Neuron Polarization

Neuron polarization is defined as the formation of an axon or “axogenesis”. Embryonic hippocampal cells are an ideal model to study this process because these neurons polarize in culture by determining a single axon and several dendrites [6,13]. This event spontaneously occurs in culture during the first 48-72 h, during which neurons follow well-defined stages, from stage 1 where cells are unpolarized, to stage 3 where cells establish an axon [13,14] (Figure 3).

In stage 1, attached neurons form motile lamellipodia around the cell body; in stage 2, there is an outgrowth of minor processes or neurites, which extend about 10-15 µm after a couple of hours in culture. These neurites are very motile but do not exhibit much net elongation, but rather continuously extend and
retract. After 1-2 days in culture, stage 3 cells form a single axon from the growing neurites. At this point, one of the neurites has an abrupt increase in growth rate (5 - 10 times the rate of the other neurites), which finally becomes the axon (polarized cell). Dotti et al. [13] reported that all cells observed in their studies followed this same pattern. Finally, in stage 4 cells, dendrites begin growing and developing from the minor neurites. This process happens after 4 days in culture (2-3 days after axon formation). Stage 5 neurons are fully-mature neurons that continue growing axons and dendrites for several weeks and ultimately form synapses.

**Figure 2.3.** Neuron polarization. Neurons undergo a series of stages from a completely rounded morphology, to a mature neuron with dendrites and a single axon. Figure adapted from [14].

Numerous studies have focused on understanding the underlying mechanisms that determine the polarization phenomenon from similar growing neurites, and major molecules such as Cdc42, PIP₃, GSK-3β and PAR-3 have been identified as playing a critical role [15-18]. It has been proposed that neuronal polarity is determined as a consequence of both a positive feedback loop at the growth cone that enhances growth by regulating actin dynamics, and a negative feedback loop that propagates from growth cones throughout the neuron with long-range negative signals, such as cAMP/PKA pathways, to inhibit the other neurites from growing further [12,19].
In the positive feedback, phosphatidylinositol-3-OH-kinase (PI 3 kinase) and the GTPases Cdc42 and Rac1 play a major role (Figure 2.4). PI 3 kinase activity has been shown to be localized at the neurite that becomes the axon [12]. PI 3 kinase activation acts on Rap1, which itself activates Cdc42, and later activates Rac1. These GTPases are known to control actin dynamics and assembly, essential for axon growth [3]. In addition, it is known that Rac1 activates back PI 3 kinase, which ultimately creates the feedback loop that determines rapid axon outgrowth during polarization. PI 3 kinase also activates other essential molecules that participate in microtubule assembly, also critical for growth [19].

**Figure 2.4.** Intracellular positive feedback loop for neuron polarization. Extracellular cues activate PI 3 kinase, which further activates the GTPases Cdc42 and Rac1. Rac1 activates back PI 3 kinase, which creates the positive feedback loop for axon rapid growth. Figure modified from [19].
The majority of investigations have focused on the identification of intracellular pathways that determine polarization in the absence of external signals. The environmental signals that can effectively influence polarization have been studied to a lesser extent. Biochemical cues such as laminin [20] and NGF [21] increase the number of hippocampal cells in stage 3 (i.e., polarized cells) during the first 24 h in culture. Additionally, physical cues such as tension [22] and topographical features [23] also influence the directionality or rate of polarization. However, combination of contact guidance and immobilized growth factors has not been explored before for polarization mechanisms and axon elongation in neurons.

2.1.2.3 Axon Guidance

Although we did not explore axon guidance in this investigation, this aspect will be investigated in future studies as explained in Chapter 9. The pathfinding mechanism of growing axons is guided by numerous molecules discussed in more detail in the following sections. The integration of these multiple attractive or repulsive signals at the growth cone determines axon trajectories to establish connectivity. There are several classical models to understand axon guidance. The most important ones are commissural axons in the spinal cord and retinal ganglion cells in the tectum (amphibians and chickens) or superior colliculus (mammals) [3].

During embryogenesis, commissural neurons have their cell bodies in the dorsal neural tube, and their axons project ventrally toward the floor plate located in the ventral midline. Once axons reach the floor plate and cross the midline, these turn abruptly to follow a longitudinal orientation [2,8] (Figure 2.5A). The floor plate attracts axons initially by releasing chemoattractant molecules (e.g., netrins). Once axons reach the floor plate and cross the midline, these are
prevented from crossing again by the presence of chemorepulsive molecules (e.g., slits).

Retinal ganglion cells are located in the outer layer of the retina. The axons from these neurons also follow a well-defined trajectory by projecting toward the optic disk, followed by leaving the retina as part of the optic nerve. After this, some of the retinal axons project ipsilaterally (same side), while others cross to project to the contralateral (opposite side) superior colliculus (Figure 2.5B). Repulsive cues such as ephrins are critical for retinal projections.

![Figure 2.5](image)

**Figure 2.5.** Classical models for axon pathfinding mechanisms. A) Commissural axon trajectories in the developing spinal cord. Axons are initially attracted by the floor plate. After crossing the ventral midline, axons turn abruptly to follow a longitudinal orientation. B) The retinotectal pathway for retinal axons. Axons leaving the retina project to either the same side (green) or the opposite side (orange) of the tectum. Figure adapted from [3].

### 2.2 Extracellular Stimuli

There are multiple extracellular factors that influence cell behavior. Here, the most studied of these stimuli, with special emphasis on neuron responses, are described. Although only a subset of these stimuli have actually been investigated in this thesis, many of the other stimuli could be investigated and implemented in the future to further optimize materials for tissue engineering.
2.2.1 Chemical Stimuli

Chemical guidance is mediated by specific receptor-ligand complexes in the cell membrane. Although we only investigated the effect of NGF for biomaterial modification and laminin as a positive control in experiments, we provide here a broader summary of the most important families of molecules that promote or control axon outgrowth and/or steering. These proteins could be used in future studies for modification of other biomaterials.

2.2.1.1 Neurotrophins

Neurotrophins, a family of growth factors, have been investigated extensively because of trophic and chemotactic effects, in addition to their roles in cell survival and differentiation [23-25]. There are six members of this family of growth factors: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, NT-4, NT-5 and NT-6 [24]. Cells respond to these proteins by expression of particular receptors on the cellular membrane. The low affinity receptor p75NTR binds to all neurotrophins with the same affinity, whereas highly specific receptors bind to each one of the neurotrophins: TrkA binds to NGF, TrkB binds to BDNF and NT-4/5 and NT-3 bind to TrkC [24,27]. After binding to high-affinity receptors, these membrane proteins dimerize and phosphorylate tyrosines to initiate the intracellular cascades that transduce the signal to the remainder of the cell. There are several signaling cascades that are activated after receptor phosphorylation such as Ras/extracellular signal regulated kinase (ERK) pathway (for differentiation), phosphatidylinositol-3-OH-kinase (PI3 K) pathway (for survival and neurite extension) and phospholipase C-γ1 (PLC-γ1) pathway (Ca^{2+} signals for multiple responses) [27].
2.2.1.2 Netrins

Netrins are proteins primarily involved in the guidance of commissural axons in the developing spinal cord [28,29]. Netrins are secreted proteins with a basic domain at the C-terminus that binds to ECM components. These proteins share a sequence homology with laminin. The netrin receptors are the UNC-40/DCC (deleted in colorectal cancer) family and Unc5H-1,-2 and -3 [30].

Interestingly, netrins can be both attractive and repulsive depending on intracellular levels of different molecules such as cAMP, cGMP and calcium [32]. In general, high levels of cAMP or cGMP and low levels of calcium enhances axon growth [33,24]. Also, this double-function relates to receptor binding, being attractive when netrins bind to DCC receptors and repulsive when these bind to Unc5H [30]. Netrins can guide a great variety of axons including hippocampal and cortical axons, retinal ganglion cells and commissural axons in the spinal cord. This guidance can be long-range (i.e., few millimeters), or short range [31].

2.2.1.3 Semaphorins and Slits

Semaphorins and slits are a large family (~30 members) of both secreted and cell surface molecules that are classified into 8 classes. These proteins all share a common "sema" domain, which dictates the specific binding of semaphorins. Semaphorins bind predominantly to two different receptors: plexins and neuropilins. Neuropilins and plexins can combine to form receptor complexes where neuropilins are the binding domain (this receptor does not have a cytoplasmatic domain) and plexins are the transduction domain [8, 30,31].

Semaphorins are repulsive cues that promote growth cone collapse. However, semaphorins can be attractive in some axons depending on
intracellular levels of second messengers. The best studied semaphorin is Sema 3A, which binds to neuropilin-1 and is exclusively repulsive [30,31].

Slits are secreted molecules that bind to Robo receptors (“roundabout”), which have cytoplasm domains for transduction. Slits appear to be exclusively repulsive for all growth cones, although slit-2 can also produce elongation and branching in dorsal root ganglia [8, 31].

2.2.1.4 Extracellular Matrix (ECM) Components

ECM components comprise the extracellular structure that surrounds cells in tissue. The ECM has a variety of functions including mechanical support for cells, cell adhesion, growth factor and hormone binding, and multiple signaling cascades that influence proliferation, differentiation or migration. There are two types of ECM components: glycosaminoglycans (GAG) (e.g., hyaluronic acid, chondroitin sulfate) and fibrous proteins (e.g., collagen, laminin, fibronectin) [35].

Integrins are the receptors that bind to ECM components. Each integrin is formed by α and β subunits. Different receptors are formed by permutations of the α and β subunits that specifically bind to different ECM molecules. Cell focal adhesions are formed by integrins, ECM molecules, focal adhesion kinases (FAK) and actin-binding proteins such as vinculin and talin [35].

Some of the important ECM components involved in axon or neurite extension are laminin, collagen and fibronectin. Laminin is an adhesive protein that is part of the basal lamina. This molecule has a cross-shaped form with three polypeptides that bind to other ECM components and integrins (Figure 2.6). Laminin has profound effects on neurons, promoting neuron adhesion, polarization and neurite elongation [20,36]. Collagens are a family of fibrous
proteins, mainly secreted by connective tissue. Collagen is a triple-stranded helical structure that arranges into cordlike structures. After secretion, collagen forms microfibrils. Fibronectins are glycoproteins formed by polypeptide dimers. Fibronectin has binding sites for other ECM components and for integrins. In particular, the fibronectin binding site for cell receptors includes the well-known tripeptide sequence formed by Arg-Gly-Asp (RGD) [35].

![Diagram of Laminin Structure](image)

**Figure 2.6.** Laminin structure. Modified from [63].

### 2.2.2 Physical Stimuli

The most studied physical stimulus is contact guidance, which is mediated by surface topography. In addition, other physical stimuli such as tension and substrate stiffness are also modulators of cell behavior.

#### 2.2.2.1 Contact Guidance

Contact guidance has been recognized as a guidance mechanism for neurons for many years. Observations in developmental studies acknowledged the presence of natural physical cues provided by glial cells to migrating neurons
to the central nervous system, as part of a complex guidance system with numerous cues [37-39]. In an attempt to mimic such physical signals, artificial substrata have been designed with microstructures, generally channels with defined pitches, grooves and ridges that stimulate nerve cells to grow, in the majority of the cases, in a parallel orientation to the channel (Figure 2.7).

**Figure 2.7.** Schematic of neurons growing on contact guidance cues.

A wide range of patterns effectively influence neurite orientation and length. Specifically, depths between 15 nm and 4 \( \mu \text{m} \), and widths ranging from 1 \( \mu \text{m} \) to 25 \( \mu \text{m} \) influence both alignment and growth rate of neurites [40-42]. The more evident effects have been observed for depths larger than 1 \( \mu \text{m} \) [40,41]. The degree of alignment is inversely proportional to groove width, but the depth of the channel exerts a larger effect than the width itself (e.g., deeper channels produce more orientation) [40]. Contact guidance is not necessarily a product of physical constraint. Depths only larger than 5 \( \mu \text{m} \) really represent impassable obstacles for cells, but for smaller distances the alignment is a product of cellular recognition [42].

The mechanism by which neurons respond to topographical features is not completely understood. It has been suggested that the higher alignment is caused by redistribution of focal adhesions in an attempt to minimize cytoskeleton distortion [40], and activation of signal transduction pathways (e.g., increase in tyrosine phosphorylation) [41]. Some authors have highlighted the role of secondary messengers, calcium influx, and proteins that modulate actin
Finally, investigations on fibroblasts have evaluated the influence of physical topography on nuclear alignment and gene regulation [44].

2.2.2.2 Stress and Substrate Stiffness

Stress forces such as shear flow can influence cell behavior [reviewed in 45]. For instance, focal adhesions can reorient under flow or stretched substrates, causing stress fiber formation and increased ECM production. Extension of the cell membrane with micropipettes influence nuclear alignment. Also, tension applied with a micropipette to neurites of stage 2 hippocampal cells induces axon formation [22]. Similarly, tension created by rotating magnetic beads attached to integrins correlates to gene expression and recruitment of ribosomes and mRNA.

Cells can also respond to substrate stiffness and elasticity by adjusting adhesion and cytoskeleton arrangements [reviewed in 46]. Soft substrates with small elastic moduli $E$ ($E \sim 1$ kPa) induce dynamic adhesion complexes, whereas stiff materials ($E \sim 30-100$ kPa) induce stable focal adhesions. In addition, tyrosine phosphorylation is enhanced on stiffer substrates. In neurons, softer surfaces produce more neurite branching and down-regulation of integrins. Molecular mechanisms that control cell responses to stiffness are not well understood. Hypotheses include activation of stress-activated ion channels that create calcium transients. Similarly, substrate elasticity has been correlated to GTPase activity such as Rac and Rho [46].

2.2.3 Cellular Stimuli (Glia Cells)

Cell-cell interactions play a fundamental role in neuron development. The importance of glial cells in mediating axon guidance is well documented. There
are three key types of glial cells in mature mammals: astrocytes and oligondendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system [47].

Glia cells are involved in many processes of neuronal development and neuron migration. A classical example is the radial migration of newborn neurons in the neocortex in the embryo. Neuroepithelial cells called radial glia serve as scaffolds that precisely guide the migration of ~90% of neurons that reach their final destination in the multiple layers of the cortex [4,48] (Figure 2.8). These glia are believed to be precursors of astrocytes in the mature brain.

![Figure 2.8](image)

**Figure 2.8.** Migration of neurons on glia and development of cerebral cortex. Neurons migrate along radial glia to reach their final location in the different layers of the cortex and create connectivity. Figure adapted from [4].

Glia are also intermediate guideposts and target cells for elongating axons [47]. For example, peripheral glia can migrate ahead of axon growth cones to position themselves in an array that guides sensory or motor axons leaving or entering the CNS. Additionally, glia can release chemotactic factors that influence both neuron survival and axon steering (e.g., netrins and slits) [47,48]. Glia are also known to form inhibitory boundaries that prevent axons from crossing or deviating [47]. This is the case of astrocytes that produce ECM
components such as tenascin and chondroitin sulfate, which are highly restrictive of axon growth. These restrictive areas form boundaries that help axon guidance in different locations such as the retina, striatum and spinal cord [49].

In addition to the role in development, glia are critical players in nerve injuries [48]. In the CNS, glial scar formed by astrocytes is one of the major inhibitors of regeneration. Molecules such as Nogo, a membrane protein associated with oligodendrocytes, are also potent inhibitors of axon regrowth. In contrast, Schwann cells in the PNS are important in regeneration by providing “bridges” that axons follow to create new connections [50].

2.2.4 Electrical Stimuli

The effect of electric fields in neuronal behavior is less documented and understood than the other cues described above. McCaig and Rajnicek described in two early comprehensive reviews the importance of electrical fields in neurite growth, orientation and nerve regeneration [51,52]. In these reviews, the following aspects of electrical stimulation are highlighted:

- There is an increase in neurite growth rate in the presence of electric fields. Neurites near the cathode grow 3-8 times faster than neurites near the anode in fields of 70-140 mV/mm.

- There is a distinctive neurite growth orientation toward the cathode in electric fields, known as galvanotropism. There is a clear orientation of the neurites with threshold field values from 7-250 mV/mm. This effect is found both for steady and pulsed fields. Additionally, retraction of neurites near the anode is also observed.
Electric fields affect growth cone morphology. Growth cones near the cathode present more filopodia than those facing the anode. In addition, twice as many filopodia in a single growth cone extend toward the cathode than the anode.

Electric fields influence neurite branching. Neurons oriented toward the cathode have twice as many lateral projections. This is also observed with localized electric fields provided by a micropipette.

Several mechanisms for electric field effects on neural behavior have been proposed. Possible redistribution of membrane proteins in electrical fields has been investigated [53]. In this case, proteins with negative charges, such as concavalin A and acetylcholine receptors, accumulate in the cathode-facing side of the cells [53]. Preferential distribution of receptors could have a profound effect on adhesion, which might explain the change in direction of growth. Similarly, this receptor redistribution could affect the assembly of cytoskeleton components.

Another effect of electric fields is the voltage drop across the membrane. For field strengths between 1 to 10 V/cm, a depolarization of 0.5-5 mV was reported [53,54]. This mechanism could enhance an electrophoretic movement of specific molecules within the growth cone, such as Ca\(^{2+}\), as a consequence of activation of voltage-activated channels. In a similar way, intracellular potential drops of about 0.01-1 \(\mu\)V across the cells were detected in the presence of external fields, which could produce preferential accumulation of charged components essential for growth [53].

The majority of studies described above used salt bridges connected to saline reservoirs and electrodes to apply electric fields. However, electrical stimulation has also been achieved with conductive substrates and by passing current through the material. One example of this stimulation is the use of
conducting polymers such as polypyrrole. This topic will be described in detail in Chapter 3.

2.2.5 Interactions Among Extracellular Cues

Although each described cue in the previous sections is important for neural development and growth, interactions among multiple stimuli could be more relevant to effectively influence neuron behavior.

McCaig suggested possible interactions between electric fields and chemical molecules. For example, he suggested that molecules such as laminin could be distributed according to their charge and endogenous electric fields. Because of this, axon orientation in electric fields was shown to depend on substrate properties, producing orientation toward the cathode on laminin surfaces, and toward the anode on polylysine surfaces [55]. Experiments with simultaneous contact guidance cues and electric fields showed that neurite orientation was mostly dominated by the direction of the electric field [56]. In contrast, adhesive cues such as laminin stripes were preferred over simultaneous electric fields for neurite alignment [57].

Britland et al. published two investigations on interactions between topographical cues and adhesive tracks [58,59]. Stripes of adhesive aminosilanes [58] or laminin [59] were orthogonally placed on quartz microchannels. Cells were preferentially aligned along adhesive tracks over the microchannels, depending on groove dimensions. More recently, a similar study by Charest et al. [60] created microchannels that were orthogonal to fibronectin tracks. In this case, osteoblasts were cultured and found to preferentially align with the physical structures rather than the chemical tracks.
Other studies have also analyzed potential interactions among multiple extracellular cues. Two publications by Miller et al. analyzed neurite orientation in the presence of Schwann cells, laminin and microchannels [61,62]. In these studies, polylactic acid microchannels were modified with laminin and seeded with Schwann cells. The authors found that this combination promotes higher neurite alignment of neurons and accelerates neurite outgrowth [61].

In summary, multiple extracellular cues create more complex environments for understanding cell behavior, which is more representative of an in vivo scenario. In this thesis, we focused on presenting simultaneous stimuli to neurons to further understand and control polarization and axon growth.

2.3 References


Chapter 3
Material Modification: Protein Immobilization, Topography and Polypyrrole

Introduction

There are numerous extracellular cues as described in Chapter 2, that can be designed and engineered to control neuron behavior. We have focused on three of these stimuli: 1) chemical guidance by growth factors, in particular NGF, 2) contact guidance by microchannels, and 3) electrical stimulation by conducting polymers, in particular polypyrrole. This chapter explains in detail the main characteristics of each one of these cues in the context of modification of biomaterial surfaces.

3.1 Materials Modified with a Chemical Stimulus: Nerve Growth Factor

3.1.1 Nerve Growth Factor (NGF) Characteristics

NGF is the most studied and characterized neurotrophin. The biologically active NGF (β subunit) has a molecular weight of 26 kDa, and consists of two 13 kDa polypeptide chains [1]. Neurons that are responsive to NGF express two types of receptors on the cell membrane, a high affinity (TrkA) and a low affinity receptor (p75NTR) [1,2]. TrkA is a transmembrane protein with a receptor tyrosine kinase for NGF. P75NTR is a transmembrane glycoprotein that binds to all neurotrophins with the same affinity [1]. NGF intracellular signaling is broad and quite complex [1]. Appendix A summarizes the most important pathways.

NGF signaling has profound effects on differentiation, survival, apoptosis and neurite outgrowth [1-7]. In addition, NGF is also chemotactic when presented in gradients [8-10], which has been related to increased adhesiveness of
filopodia on the proximal side to the NGF source and elevation of cAMP and calcium [9,11].

NGF also plays an important role in regeneration. After a peripheral nerve injury, the body initiates a mechanism for regeneration mediated by glia and inflammatory cells. In particular, Schwann cells proliferate and release growth factors, including NGF. The complex role of NGF in regeneration is not completely clear, but it increases the number and myelination of regenerating axons [2,12]. Because of this evidence, the presence of NGF for nerve repair therapies in both the PNS and CNS might be crucial [12], and attempts to deliver it by different methods have been explored [13-15].

3.1.2 Immobilization of NGF in Neural Engineering Applications

Tissue engineering and implant strategies often include growth factors as stimulating signals for regeneration. Some studies encapsulate these proteins in degradable polymers that release the protein over time in a diffusible form [15]. Alternatively, immobilization of growth factors to the substrate has also been applied in other investigations. Several growth factors such as insulin [16], epidermal growth factor (EGF) [17], VEGF [18] and NGF [19] have been conjugated to different materials for enhancing cell responses. The following sections describe general techniques for immobilization of proteins and a comprehensive summary of investigations on immobilized NGF.

3.1.2.1 Protein Patterning and Immobilization

There are three types of protein immobilization: 1) protein adsorption, 2) physical entrapment, and 3) covalent attachment [20] (Figure 3.1). Protein adsorption is driven by attractive forces such as electrostatic, hydrophobic and
Van der Waals forces [20,21]. Physical entrapment is mediated by barrier systems such as hydrogels and dispersed or matrix systems [20]. Finally, covalent attachment can be performed by a wide variety of reactions with different functional groups.

Protein covalent immobilization requires reactive functional groups (e.g., -OH, -NH₂, COOH) on the surface of the material, which can be introduced by different techniques such as photochemical fixation, plasma discharge and chemical modification [20]. A great variety of reactive compounds have been investigated for protein immobilization including isothiocyanates, glutaraldehydes, carbodiimides, and hydroxysuccinimides. A summary of the most important methods can be found in [20].

Various techniques such as self-assembled monolayers (SAMs) and photochemical techniques are used to pattern proteins. For SAMs, patterned silanes or alkane thiols are used to bind proteins to silica or metal surfaces. The patterns are obtained in conjunction with soft lithography or conventional photolithographic methods [21]. Photo-chemistries use molecules that are activated upon UV irradiation to create covalent bonds with the surfaces. Photomasks are used during the exposure step to create protein patterns. The most common photochemical methods use arylazides, nitrobenzyl, aryl diazirine and benzophenone [21], as illustrated in Figure 3.2.
In the aryazide chemistry, which is used for NGF immobilization in the following chapters, arylazido-containing compounds react nonspecifically with UV light by creating singlet nitrenes that undergo insertion into C-H, N-H and other bonds [22,23] (see Appendix B for scheme of reactions). Using this method, proteins have been fixed to many substrates such as poly(vinyl alcohol), polystyrene, poly(ethylene terephthalate) and chitosan [24-27].

Figure 3.2. Photochemistry reactions for protein immobilization. Chemical groups are activated upon UV irradiation to form radical intermediates that react with substrates as illustrated in the schematic. Chemical structures of the most common photo-activated grups for protein immobilization. Figure modified from [21].
3.1.2.2 Studies on Immobilized NGF

NGF has been substrate-immobilized in several investigations since 1973 using a variety of methods. Three early studies [28-30] showed that substrate-bound NGF is capable of inducing outgrowth and guidance of neurites. Frazier et al. [28] immobilized NGF to Sepharose beads and showed preservation of activity with neurite outgrowth assays. Gundersen showed neurite extension and orientation of chick DRG on surface patterns of absorbed NGF [29]. Sandrock et al. showed neurite growth on cryostat sections with adsorbed NGF [30].

Pettmann et al. [31] used anchorage of NGF to nitrocellulose paper by Western blotting. Ciliary, dorsal root and sympathetic ganglia neurons were subsequently cultured on the paper containing the NGF bands and shown to extend neurites only on the immobilized NGF. This study further proved that substrate-bound NGF is active and has both survival and neurite outgrowth effects on neurons. The authors also discussed the relevance of such results to an in vivo scenario, where NGF could be presented to cells in an anchorage-dependent form with advantages such as increased stability and a more localized action.

Two papers by Gallo et al. [32,33] covalently-immobilized NGF on polystyrene beads. These studies demonstrated that growth cones of chick DRG neurons turned and migrated when in contact with the NGF-coated beads. Furthermore, the immobilized NGF also induced collateral sprouting and branching of axons. Importantly, the authors showed that responses to the immobilized protein were mediated by TrkA receptors through a PI 3 kinase-dependent pathway.
A study involving the covalent immobilization of NGF using an arylazide photochemistry [34] showed neurite extension of PC12 cells on immobilized NGF patterns. A more recent publication by Kapur and Shoichet [35] using the same photochemistry further demonstrated that chemically-bound NGF is active and induces neurite extension from PC12 cells. This same group extended the concept to the creation of immobilized gradients of NGF on hydrogels using a “gradient maker” [36]. The results from this publication showed that immobilized NGF was not only active, but also capable or guiding neurite extension from PC12 cells when presented as an immobilized gradient. The same technique and methods were again used in another publication from the same group with primary chick DRG neurons [37].

An important paper by MacInnis et al. [38] illustrated that immobilized NGF on cross-linked beads increases the phosphorylation of TrkA receptors and Akt (related to PI 3 kinase pathway), but did not increase phosphorylation of MAP kinase in sympathetic neurons. These results suggest that survival pathways do not depend on endocytosis. NGF has been also presented to Schwann cells within gelatin membranes modified with immobilized NGF [39].

Combined, these studies provide strong evidence that immobilization of NGF is feasible and that the protein retains activity in a substrate-bound state.

3.2 Materials Modified with a Physical Stimulus: Artificial Microchannels

Contact guidance in the form of micro- and nanostructures on the surfaces of materials influences neurite outgrowth. In the following sections, contact guidance by microchannels is discussed, beginning with a summary of microfabrication techniques.
3.2.1 Microfabrication Techniques

Photolithography is the most conventional technique in microfabrication, especially for the integrated circuit industry. The basic process consists of applying a UV-sensitive photoresist to a substrate, mostly silicon, which is subsequently exposed to UV light by using a mask as a template. The exposed regions of the photoresist become either harder or more soluble depending on the type of photoresist, creating patterns after dissolution with solvents (i.e., development) (Figure 3.3) [40,41]. The development of the resist can be followed by numerous techniques depending on the specific process, such as wet and dry etching (Figure 3.4), lift-off, and additive techniques [40].

Another technique for the fabrication of micropatterns is electron beam (e-beam) lithography (Figure 3.5). This direct-writing technique creates structures on photoresists that are sensitive to high-energy electron irradiation provided by a computer-controlled electron gun. The exposed resist can be subsequently removed for the formation of the patterns. E-beam lithography does not require a physical mask, can be used to create numerous shapes and designs, can achieve very high resolutions, but is time-consuming and expensive. However,

![Figure 3.3](image-url) Photolithographic processes. A photoresist is exposed to irradiation through a photomask. The resist can either polymerize and harden upon exposure (negative), or become soluble (positive). Figure adapted from [41].

Figure 3.5. Image of electron beam lithography setup. The electron beam is directed from the source (top) through the mask (middle) and onto the substrate (bottom) where the resist is exposed. The developed resist forms the pattern.
this technique is required for the fabrication of photomasks, which are essential for all photolithographic techniques [40,42].

![Figure 3.4](image)

**Figure 3.4.** Etching techniques. After photolithography is performed, pattern transfer techniques are used to transfer the pattern to the underlying silicon wafer. This can be formed by etching, which gives different profiles depending on the type of etching. Figure adapted from [41].

![Figure 3.5](image)

**Figure 3.5.** Electron beam lithography. An e-beam resist is exposed with high-energy electron irradiation. The movement and writing of the electron gun is controlled by a computer according to the user’s design. Figure modified from [42].

Soft lithography is a novel technique also used for microfabrication purposes. The term “soft” refers to the use of soft polymers, i.e., elastomers, for creating patterns, instead of conventional silicon [43]. Poly(dimethyl siloxane) (PDMS) is a polymer often used in this technique, composed of repeating OSi(CH₃)₂ units. The basic process in soft lithography consists of several steps.
The first procedure is the fabrication of a master using conventional UV photolithography. For this, a chromium mask with a defined pattern is used for exposing a photoresist (usually SU-8) previously spun onto silicon. This master is subsequently used as a mold for performing replica molding by pouring a prepolymer mixture, curing and obtaining the final PDMS piece or “stamp” [44,45] (Figure 3.6). Most of the applications of soft lithography use the PDMS as a stamp for patterning other materials [43], but other applications directly use the PDMS itself, such as in microfluidics [45]. The master can be used numerous times for obtaining PDMS structures, which makes this technique easy to use, inexpensive and adaptable to rapid prototyping. Additionally, PDMS is non-toxic for cells, allowing the extension of soft lithography to biological applications [46]. Soft and e-beam lithography were used in this investigation for the fabrication of microchannels for contact guidance.

Figure 3.6. Soft lithography techniques. A master is created using conventional photolithographic processes. The patterns are transferred to the elastomer PDMS by replica molding. Figure adapted from [46].
3.2.2 Neurons on Artificial Microchannels

Microchannels are the most common designed features to control neurite alignment and growth (Figure 3.7). Hirono et al. [47] reported the behavior of DRG neurons on microchannels (0.7-10 µm wide, 0.1-2 µm deep) fabricated in quartz using conventional photolithographic techniques. This study demonstrated that DRG neurites grow axially along the structures and on the bottom grooves of the microchannels. The alignment of neurites was found to be a function of depth and width of the topographical features, having more alignment for larger depths and widths. The authors also highlighted that effects of topography were mostly mechanical as coating of the microchannels with different chemical molecules did not influence neurite growth.

![Figure 3.7](image.png)

**Figure 3.7.** Microchannel characteristics and dimensions.

A well-known study on topographical effects on cells was performed by Clark et al. [48]. The effect of grooved substrates (4-24 µm wide, 0.2-1.9 µm deep) on three cell types, including chick embryo cerebral neurons, was analyzed. The results showed that cell alignment depend on pattern dimensions, but groove depth has the most overall impact. The authors discussed some possible mechanisms for topographical guidance, suggesting the effects of confinement of focal adhesions and distortion of the cytoskeleton.
Perpendicular contact guidance (i.e., alignment of neurites perpendicular to the axis of the microchannels) was reported by Nagata et al. [49]. CNS neuroblasts but not PNS neurons, were able to grow neurites both in perpendicular and parallel directions to 1 µm microchannels with depths between 0.3 and 0.8 µm. The authors emphasized that this phenomenon seemed to be unique to CNS neurons, and that the 1 µm structures mimic tightly-aligned neurite bundles provided by other cells in the body.

In addition to microtopography, neuron behavior on nanometer-scale structures has also been studied. For example, Clark et al. [50], studied “ultrafine topography” with dimensions of 130 nm in width and depths between 100-400 nm. In this study, chick embryo cerebral neurons were not affected by grooves. In contrast, a more recent study with similar dimensions on poly(methyl methacrylate) patterns (300 nm deep, 100-400 nm wide) [51] showed that mouse sympathetic and sensory ganglia neurons exhibit contact guidance mechanisms, and that neurites prefer to grow on top of ridges rather than within grooves.

Curtis et al. reported another effect of microchannels on neuron behavior in addition to alignment [52]. This publication reported that DRG neurons had a seven-fold increase in speed of neurite extension on 5 µm grooves with respect to flat surfaces. Also, growth cones were much more motile on grooved substrates, moving or extending 92% of the time with respect to 13% on flat surfaces. Another effect of topography was recently investigated by Foley et al., who investigated the effect of ridges between 70-1900 nm on neurite initiation in PC12 cells [55]. They found that ridges between 70 and 250 nm significantly increased “neuritogenesis”, but only when cells were cultured in low concentrations of NGF.
Two important papers by Rajnicek et al. [53,54] used both *Xenopus* and hippocampal neurons to study the effect of microchannels on axon growth. The patterns were fabricated in quartz with dimensions of 14-1100 nm in depth and 1-4 \( \mu m \) in width. These studies found that *Xenopus* axons grow primarily in a parallel orientation, whereas hippocampal axons grow parallel to deep grooves, but perpendicular to shallow patterns. The authors emphasized that contact guidance by patterns less than 5 \( \mu m \) deep is not only an effect of physical constraint, but rather an effect of stimulation by the physical topography to extend axons along the grooves. Patterns that are deeper than 5 \( \mu m \) actually constraint and restrict axon growth, which produce alignment as a product of containment but not of cell recognition. These authors also performed extensive analysis of possible intracellular molecules that mediate the responses to topography. They found that perpendicular alignment is highly affected by calcium channel blockers and protein kinase C inhibitors.

Although Rajnicek et al. suggested that topographical features deeper than 5 \( \mu m \) would be more of a barrier than a true stimulus, there have been a couple of recent investigations on deep microchannels. Mahoney et al. [56] studied neurite alignment of PC12 cells on polyimide microchannels 11 \( \mu m \) deep and 20-60 \( \mu m \) wide. They found that these structures impact neurite direction, increase length and reduce the number of neurites and neuronal architecture. An interesting investigation by Goldner et al. [57] used very deep PDMS microchannels (50 \( \mu m \) deep, 200 \( \mu m \) plateau wide, 30 \( \mu m \) groove wide) to show that neurons were able to bridge across these grooves. They showed by time-lapse images that neurons that were initially in grooves extended neurites to the walls, pulled themselves up until the neurons were suspended between ridges, and finally pulled the cell soma to the plateau of the patterns.
3.3 Materials with an Electrical Stimulus: Polypyrrole

Polypyrrole (PPy) is a biocompatible electrically-conductive polymer that has been used for neural prosthetics, in addition to in vitro and in vivo studies for nerve regeneration and other tissue engineering applications. In the following sections PPy polymerization methods, chemical modification and cell studies on this polymer are described.

3.3.1 PPy Polymerization

PPy is commonly polymerized either chemically or electrochemically. For chemical polymerization, a strong oxidizing agent such as FeCl₃ is mixed with the pyrrole monomer, which produces charged pyrrole rings that react with other rings to continue the polymerization. The positively charged radicals are electrically neutralized with anions from the oxidant solution [58]. Although chemical polymerization yields larger quantities of polymer, electropolymerization is usually preferred for having better control over material properties.

PPy electropolymerization produces films of the polymer on conductive electrodes. For this reaction, electrodeposition occurs at a positively charged working electrode, where monomer units adsorb and oxidize to form pyrrole cationic radicals. These cations couple via a condensation reaction with other cations or monomers from the solution. As the film grows, radicals are continuously being produced on the surface of the polymer, which allows the polymerization to continue (Figure 3.8). To maintain electrical charge balance, negatively-charged counterions are inserted during the polymerization of pyrrole cations, which is known as doping [59-61].
Figure 3.8. Electropolymerization of PPy. Cationic radicals are produced on the surface of the working electrode during the electropolymerization, which condense with other cations or monomers to produce the polymer. Figure modified from [59].

The electropolymerization setup usually consists of a standard three electrode configuration (working, counter and reference electrodes) in an electrochemical bath of monomer and an electrolyte in an appropriate solvent (Figure 3.9). This polymerization can be performed potentiostatically (constant voltage) or galvanostatically (constant current) [61]. The working electrode is the
substrate for the electro-deposition and common inert materials are Pt, Au, indium tin oxide (ITO) and stainless steel. The counter electrode is a metallic foil of Pt, Au or Ni. Finally, the reference electrode can be a saturated calomel electrode or Ag/AgCl electrode [61].

PPy is a conductive polymer because of its highly delocalized \( \pi \)-system in the backbone, which allows electrons to migrate throughout the polymer chains and transfer charge to other chains by electron “hopping” [59,60].

![Figure 3.9](image)

**Figure 3.9.** Electropolymerization setup with a three electrode configuration. Figure modified from [61].

### 3.3.2 PPy Modification for Biological Applications

PPy has been extensively modified for biological applications. There are three types of chemical modification: 1) surface modification post polymerization, 2) dopant modification during polymerization, and 3) monomer modification to incorporate other functional groups, such as amine groups (Figure 3.10).
Figure 3.10. PPy modification for biological modifications. A) Surface modification; B) dopant modification; C) monomer modification (PPy* = polymer with modified pyrrole monomer).

3.3.2.1 Surface Modification

Surface modification techniques use chemical approaches to modify the polymer and attach other molecules after polymerization. Cen et al. published two articles on functionalization of PPy with hyaluronic acid (HA) via grafting of acrylate groups and silanization with amine-containing silanes [62,63]. HA was subsequently immobilized using carbodiimide chemistry for the reaction of amine groups from the grafting procedure and the carboxylic groups from HA. The authors found that this surface modification of PPy preserved the conductivity (4 - 38% decrease, depending on the degree of functionalization), maintained the mechanical strength of the polymer and increased the hydrophylicity of the surface. The authors studied cell interaction with this modified polymer by culturing PC12 cells and platelets. The same group also immobilized glucose oxidase to PPy in another publication using similar approaches, showing that the enzyme retained activity and increased stability in the immobilized form [64].

In other applications, hydrogels of poly(vinyl alcohol) (PVA) and heparin were covalently immobilized to PPy using a chemistry involving grafting and oxidation of poly(ethylene glycol) acrylates to incorporate aldehyde groups on the surface of PPy [65]. These groups reacted with hydroxyl groups from PVA to produce the immobilization. The authors used this modified polymer for the release of heparin from the hydrogel with electrical stimulation.
In a more recent investigation, PPy was surface modified by reactions with the dopant [66]. Polyglutamic acid was used as a dopant during the polymerization, which introduced carboxylic groups on the PPy surface. After this, conventional carbodiimide chemistry was used to attach polylysine or laminin. The authors cultured DRG neurons and found the cells to adhere exclusively to the modified PPy.

De Giglio et al. published two articles on the modification of PPy via cysteine residues [67,68]. They found that cysteine forms covalent bonds with the pyrrole rings via the sulfydryl group. Using this technique, they attached RGD sequences to PPy and cultured osteoblasts, which exhibited increased cell adhesion on the modified PPy.

Finally, in an elegant approach, Sanghvi et al. used phage-display techniques to find a peptide that specifically bound to PPy surfaces [69]. Using this peptide, the authors immobilized RGD sequences and cultured PC12 cells, which were able to adhere to the PPy surface in serum-free conditions.

3.3.2.2 Dopant Modification

Another common technique to incorporate biological molecules to PPy is to use these directly as dopants during the electropolymerization process. Following this approach, Cui et al. incorporated biologically-relevant synthetic peptides into the polymer [70,71]. The authors found that the incorporation of these peptides as dopants was beneficial as it created very rough surfaces, which was better for electrode recording in neural probes. Neuroblastoma cells attached better on the modified polymer. In addition, neural probes coated with modified PPy were tested in vivo and shown to induce strong connection with
neural tissue. A very similar approach was recently published by Stauffer and Cui with laminin peptides [72].

Incorporation of proteins as dopants in PPy was demonstrated by Hodgson et. al, who used albumin and NGF as dopants during electropolymerization [73]. This investigation showed that these proteins could be released from PPy when the polymer was reduced by applying a voltage. The protein was completely released in less than 1 minute. The authors showed that the proteins were active after being released, as PC12 cells extended neurites in the presence of the NGF released from PPy.

Finally, incorporation of glycosaminoglycans such as HA and heparin as dopants has also been investigated [74-76]. Heparin-containing PPy was shown to support attachment of endothelial cells [74,75], whereas HA-doped PPy was tested for biocompatibility using *in vitro* and *in vivo* studies [76]. HA-doped PPy was tested for conductivity, showing a decrease in orders of magnitude [76].

### 3.3.2.3 Bulk Modification

Bulk modification of PPy has been performed to change properties such as biodegradability or to introduce functional groups in the pyrrole monomer that can be used for subsequent modification with biomolecules. For example, Rivers *et al.* [77] showed the synthesis of a conducting polymer formed by pyrrole and thiophene rings that was biocompatible and biodegradable by the incorporation of ester linkages in the polymer.

Modification of pyrrole monomer to incorporate carboxylic functional groups or succinimidyl ester has also been performed [78-80]. In these investigations, pyrrole was chemically modified to have functional groups
attached to the nitrogen in the pyrrole ring (i.e., N-substituted pyrroles). After polymerization of this modified pyrrole, immobilization of proteins was performed using carbodiimide chemistry. A recent publication by Lee et al. [81] used the same approach to create PPy with carboxylic groups that were used for the immobilization of RGD sequences. Endothelial cells showed increased adhesion to the modified polymer. One important disadvantage of these approaches is the very low conductivity of the final polymer ($\sim 10^{-4}$ S-cm$^{-1}$) [81].

### 3.3.3 Cell Studies on PPy

PPy has been extensively used for cell culture. In all studies explained in previous sections, cells were able to adhere and function normally on modified PPy. More interesting results on cell behavior have been accomplished by actually passing current thought the material. Wong et al. [82] reported that endothelial cells were able to adhere and extend on oxidized PPy. However, when the polymer was reduced to a neutral state by applying an electrical potential, cell viability and extension were significantly reduced. The authors suggested that these properties of PPy could be used to precisely control adhesion and shape of cells for different applications.

An important study by Schmidt et al. [83] demonstrated that electrical stimulation of cells cultured on PPy conducting current was feasible. In this investigation, PC12 cells were cultured on PPy films. The authors found that neurite extension was enhanced when current was passed through PPy, which demonstrated that electrically-conductive PPy could be used to enhance neuronal outgrowth. A later study using the same techniques showed that electrical stimulation of PPy enhanced protein adsorption on the surface of the material, which consequently has an indirect effect on neurite extension [84].
Various investigations have studied the biocompatibility and potential application of PPy to nerve guidance channels [83, 85-87] and neural probes [88]. These studies have concluded that PPy has good biocompatibility and minimal tissue response and inflammation during implantation, which further supports PPy as a good candidate for these applications.

In summary, many materials including PPy and PDMS can be modified on the surface with chemical cues (i.e., immobilized proteins) and physical cues (i.e., topographical features) for biological applications. In this thesis, we focused on combinations of these cues. In particular, we combined either microchannels in PDMS or PPy with immobilized NGF.

3.4 References


[59] Skotheim TA. Handbook of Conducting Polymer, Marcel Dekker, 1986.


Chapter 4
Fabrication of Microchannels for Contact Guidance

Introduction

One of the extracellular cues studied in this investigation is contact guidance by microchannels. For this type of stimulation, microfabricated features are created on the surface of the material to provide physical cues to growing neurons. As stated in Chapter 1, the goal of this investigation was to combine these physical cues with chemical cues to enhance axon extension (combinatorial strategy I, Chapter 5), and to better understand polarization mechanisms in competitive environments (competitive strategy, Chapter 8). Here, the fabrication procedure to create microchannels is described. These microchannels were fabricated in poly(dimethyl siloxane) using soft lithography methods.

4.1 Background and Motivation

Contact guidance has been recognized as a guidance mechanism for neurons for many years [1-3]. Artificial substrata are designed with structures, generally microchannels, for influencing neurite extension and alignment (discussed in Chapters 2 and 3). A wide range of pattern dimensions has been investigated, including depths between 15 nm and 4 \( \mu m \), and widths ranging from 1 \( \mu m \) to 25 \( \mu m \) [5-7].

Neurons respond to topographical features by multiple mechanisms. These include redistribution of focal adhesions in an attempt to minimize cytoskeleton distortion [5], activation of signal transduction pathways [6], calcium influx and modulation of actin [8], nuclear alignment and gene regulation [9].
Hippocampal neurons are the cell model used in the present studies for investigating the effects of topography on polarization and axon extension. Rajnicek et al. [6,7] previously used these same neurons to study the effect of microchannels on axon growth. The patterns used in that investigation were fabricated in quartz with dimensions of 14-1100 nm in depth and 1-4 µm in width. These studies demonstrated that hippocampal axons grow parallel to deep grooves, but perpendicular to shallow patterns. Based on this previous investigation, we have chosen two different widths and two different depths in the range mentioned above. These dimensions included widths of 1 and 2 µm, and depths of 400 and 800 nm.

Topographical features for contact guidance applications are fabricated with multiple microfabrication techniques (discussed in Chapter 3), including photolithography [9,10], electron beam (e-beam) lithography [9,11] and soft lithography [12-14]. Both e-beam lithography and soft lithography were used in this investigation to create microchannels in poly(dimethylsiloxane) (PDMS). PDMS is non-toxic for cells, transparent and inexpensive, which are good characteristics for biological applications [15].

4.2 Materials and Methods

4.2.1 Microfabrication

Microchannels 1 and 2 µm wide and 400 and 800 nm deep were created on PDMS using soft lithography techniques. The procedure consisted of three steps: 1) fabrication of a mask with the desired patterns, 2) fabrication of a silicon master, and 3) replica molding of the PDMS.
4.2.1.1  *Electron beam (e-beam) Lithography*

The mask was created with e-beam lithography. 4-µm-thick low temperature oxide was initially deposited on a silicon wafer (WaferWorld) using low pressure chemical vapor deposition (LPCVD). The wafer was then annealed at 1000°C for 1 h to enhance film density and etching durability. The wafers were subsequently cut into 1 cm²-pieces and cleaned by sonication in acetone, isopropyl alcohol and water. 4% poly(methyl methacrylate) PMMA resist in chlorobenzene (Microchem) was spin coated for 45 seconds and 3000 rpm, and baked for 30 minutes at 170°C. Microchannels of 1 or 2 µm were written on the PMMA resist using e-beam (Raith-50 and XL-30 SEM, LaB₆ source) with an area dose of 220 µAs/cm² and beam current of 0.2 nA. Development was done with methyl-isobutyl-ketone and isopropyl alcohol (1:3) (Sigma) for 1.5 minutes.

4.2.1.2  *Reactive Ion Etching (RIE)*

After the microchannels were written with e-beam, a thermal evaporator (Denton) was used to deposit a 100 nm chromium (R.D. Mathis) film, followed by lift-off with acetone. RIE of the SiO₂ layer was performed in an etcher (Plasma Technology) with a mixture of CHF₃ and oxygen until the desired thickness was obtained and measured with a Nanospec (Nanometrics). The Cr-coated areas were wet-etched with a mixture of ceric sulfate and nitric acid (Transene) for 2 minutes. The final master was silanized with tridecafluoro-1,1,2,2-(tetrahydrooctyl) trichlorosilane (Gelest).

4.2.1.3  *PDMS Replica Molding*

PDMS microchannels were obtained by pouring a mixture of Sylgard 184 (Dow Corning) on the master (one part curing agent per ten parts base), curing
for 24 h at room temperature and releasing the film. This step was performed multiple times for a single master.

4.2.2 Reflectance Microscopy

Images of silicon patterns were obtained using an Olympus BX51WI upright light microscope equipped for reflected and transmitted light imaging. The microscope was equipped with a color digital camera (Olympus) and interfaced with a computer. Images from the microscope were analyzed using Adobe Photoshop and Image J (NIH).

4.2.3 Scanning Electron Microscopy (SEM)

PDMS and silicon microchannels were analyzed with a LEO 1530 scanning electron microscope. Substrates were coated with a gold layer of a few angstroms thick and imaged with an acceleration voltage of 10 kV.

4.2.4 Atomic Force Microscopy (AFM)

A Digital Instruments Dimension 3100 with Nanoscope IV controller was used in tapping mode to scan the PDMS substrates and measure microchannel depth.

4.3 Results and Discussion

As illustrated in Figure 4.1, soft lithography methods were used for the creation of microchannels in PDMS. However, this technique requires fabrication of a master that serves as a mold for the subsequent replica molding of the
The fabrication of this master was performed with conventional lithographic processes.

The first step for the fabrication of the master was the design of microchannel dimensions and writing using e-beam lithography. PMMA resist was spin coated on a silicon wafer with a SiO$_2$ surface layer (5 $\mu$m). PMMA resist undergoes chain scission and bond breakage when exposed to e-beam irradiation [9], allowing the selective removal of exposed areas with organic solvents. Using this technique, patterns with desired dimensions were obtained as illustrated in Figure 4.1A. These patterns were $\sim$500 nm deep, as this was the thickness of the spin coated resist (value obtained from data provided by the resist manufacturer, Microchem).

After the patterns were on the PMMA resist, these were transferred to the underlying silicon wafer that ultimately serves as the mold. For this process, two steps are required: 1) chromium lift-off and 2) reactive ion etching. The first step creates a mask for the subsequent etching of the silicon dioxide layer. Chromium is an ideal material for masking in RIE because of its low etching rate [16]. Cr was thermally evaporated onto the PMMA-coated wafer with a final thickness of 100 nm, as directly measured during the deposition. The deposition covers the complete surface of the wafer, including the patterns. However, as the PMMA thickness is larger than the Cr thickness, it is possible to strip (i.e., dissolve) the remaining PMMA resist, leaving only the Cr patterns. This step is illustrated in Figure 4.1B, in which the patterns initially written on PMMA were transferred as Cr stripes on top of the silicon wafer.
Figure 4.1. Fabrication of microchannels. Reflectance microscope images of A) PMMA patterns on silicon wafer written with E-beam lithography; B) Cr patterns on a silicon wafer transferred with lift-off; C) patterns on SiO₂ layer on silicon wafer after RIE; D) PDMS patterns obtained by replica molding of the silicon master. Scale bar = 20 µm. All images are at the same magnification.
RIE is a technique that removes material from a target as a combination of physical and chemical processes. Plasma high-energy ions chemically react with the wafer and cause bond breakage by momentum transfer [9]. This type of etching yields square grooves, which were desired for this investigation. Other techniques such as wet etching usually produce either V-shaped or undercut profiles [9]. RIE was performed with a gas mixture of CHF₃ (66%) and oxygen (33%), which etched the entire SiO₂ surface except for the Cr-protected areas, transferring the patterns to the wafer. Microchannel depth was controlled by etching time.

The experimental SiO₂ etching rate was ~500 Å/min, as determined by measuring SiO₂ thickness with a Nanospec machine at different times during the etching (Figure 4.2 C). As observed in SEM images in Figure 4.2A and Figure 4.3A, etching of the SiO₂ layer produces smooth surfaces. In contrast, etching Si wafers without a SiO₂ layer produces much rougher surfaces (Figure 4.2B). This effect is usually a problem in dry etching techniques called “grass” or “black silicon”, produced by micro-masks from oxides or dust on the surface of the silicon [16].

After RIE was performed, patterns were finally transferred to the silicon wafer (Figure 4.1C). Following this step, the Cr film was wet-etched and the final master was silanized to prevent the PDMS from adhering. Replica molding of PDMS was performed by pouring a mixture of a prepolymer with the curing agent, curing for 24 h and detaching the PDMS films with the patterns on the surface, as illustrated in Figure 4.1D.
Figure 4.2. SEM images of silicon masters. A) Patterns on a wafer with SiO$_2$ layer; B) patterns on a wafer without SiO$_2$ layer. SiO$_2$ etching produces smoother surfaces; C) SiO$_2$ etching rate. Scale bar = 5 µm. Images are at the same magnification.

Figure 4.3 illustrates SEM images of both the final master and the PDMS replica, showing the creation of ridges on the master and the corresponding grooves on the polymer replica. The masters were used multiple times for obtaining numerous PDMS substrates for cell culture.

Microchannel depth was analyzed using AFM in tapping mode. Figure 4.4A and Figure 4.4C illustrate AFM images for 2 µm PDMS microchannels with two different depths. Figure 4.4B and Figure 4.4D show profiles obtained from the AFM images, illustrating the two different depths of the microchannels (400 and 800 nm).
Figure 4.3. SEM images of silicon master and PDMS replica. A) 2 µm microchannels on master; B) 2 µm microchannels on PDMS. Scale bar = 10 µm. Images are at the same magnification.

Figure 4.4. AFM images of PDMS microchannels. AFM image and cross-section profile of A)-B) 2 µm wide, 400 nm deep microchannels; C)-D) 2 µm wide, 800 nm deep microchannels, respectively (Images obtained by Yi Lu from Shaochen Chen's lab).
4.4 Conclusions

We used soft lithography methods for the creation of microchannels in PDMS. These microchannels were used for cell culture of neurons as explained in Chapters 5 and 8. E-beam lithography and RIE were used for the fabrication of masters on silicon wafers with a SiO₂ layer. We found that e-beam lithography was convenient for creating structures of different dimensions and shapes as required for the experiments. Dry etching of SiO₂ produce smoother surfaces than etching of Si only. Finally, PDMS microcrochannels were analyzed with SEM and AFM.

4.5 References


Chapter 5
Combinatorial Strategy I: Microtopography and Immobilized NGF

Introduction

Cell interfacing with biomaterial surfaces dictates important aspects of cell behavior. In particular, axon extension in neurons is effectively influenced by surface properties, both for the initial formation of an axon as well as for the maintenance of axon growth. We have created a novel combinatorial strategy that includes two different material surface properties that influence nerve behavior: immobilized ligands (chemical guidance) and topographical features (physical guidance). We investigated how neurons behaved on poly(dimethyl siloxane) (PDMS) surfaces decorated with biochemical and physical cues presented individually or in combination. In particular, nerve growth factor (NGF) was covalently tethered to PDMS to create a bioactive surface, and microtopography was introduced to the material in the form of microchannels (as described in Chapter 4). Embryonic hippocampal neurons were used to investigate the impact of these surface cues on polarization (i.e., axon initiation or axogenesis) and overall axon length.

5.1 Background and Motivation

Interfaces between engineered materials and cells play a critical role in biomedical applications where the interaction between cells and the material surface dictates cell performance and therefore, the success of the implanted device. Extracellular matrix (ECM) components and peptides, topographical features, support cells and growth factors [1-4] have been extensively studied for the creation of biomimetic materials that control cellular responses such as adhesion, morphology or differentiation. More recently, combinations of such
environmental cues have proven to be advantageous for targeting multiple aspects of cell behavior and improving cell responses in applications such as osteogenesis [5,6], nerve regeneration [7-9], and endothelialization [10].

With respect to neural engineering applications, patterned adhesive areas [15-17], contact guidance cues [18-20], delivery of nerve growth factors [21,22] and electrically-conductive substrates [23] have been particularly used to increase neurite outgrowth and apply this knowledge for axon regeneration after trauma [13,14]. However, whether these stimuli influence axon formation, axon elongation or both has not been extensively studied. In this study, we have focused on understanding the effect of environmental cues on axon initiation versus overall axon elongation. We also explored if different types of stimuli, such as chemical ligands that bind to specific receptors, or physical signals that are transduced by cytoskeleton tension, could preferentially affect one of these processes. We directly compared neuron responses to two different cues that were presented either individually or in combination. Immobilized NGF was used as the model chemical ligand, whereas surface microtopography in the form of microchannels was investigated as the physical stimulus. These studies were performed presenting the cues as surface properties of a designed material, which could be adapted for neural engineering applications to better design biomaterials that modulate neuron responses. For example, biomaterials used in nerve guidance conduits could be modified to present both topographical features and immobilized NGF to enhance nerve regeneration.

Physical guidance, also called contact guidance, has been recognized as a natural guidance mechanism for neurons for many years [11,24,25]. In an attempt to mimic such signals, artificial substrata have been designed with microstructures, generally microchannels (i.e., ridges and grooves) that stimulate neurons to grow aligned along the channels. A wide range of patterns effectively
influence neurite orientation and length. Specifically, depths between 15 nm and 4 µm, and widths ranging from 1 µm to 25 µm have been shown to influence both alignment [18, 26-28] and growth rate of neurites [27,29].

In addition to physical cues, chemical ligands such as growth factors are potent modulators of cell responses. Neurotrophins, a family of growth factors, have been investigated extensively because of trophic and chemotactic effects, in addition to their roles in cell survival and differentiation [30-32]. NGF is the most studied and characterized neurotrophin, known for inducing several neuron responses, including neurite outgrowth. Although some of NGF’s effects require endocytosis of the ligand-receptor complex [32], neurite outgrowth is not necessarily mediated by retrograde transport [33,38-40] and possibly controlled by localized actin polymerization [34]. Thus, NGF immobilized to substrata is effective in inducing neurite extension and even turning and sprouting [35-40]. Other growth factors including insulin, epidermal growth factor and vascular endothelial growth factor, have also been immobilized in active form on a variety of substrates [41-43]. A common approach for this protein immobilization is the use of aryazido-containing compounds, which can react nonspecifically with UV light by creating singlet nitrenes that undergo insertion into C-H, N-H and other bonds [44,45]. Using this method, proteins have been fixed to many substrates such as poly(vinyl alcohol), polystyrene, poly(ethylene terephthalate), chitosan and polypyrrole [41,42,46-49,71].

To better study axon formation responses to surface stimuli, embryonic hippocampal neurons were used because these cells are the most common and characterized model for investigating polarization [12,50]. Polarization of these neurons occurs spontaneously in culture during the first 48-72 h, during which neurons follow very well defined stages, from stage 1 where cells are unpolarized, to stage 3 where the cells establish an axon [50,51]. Numerous
studies have focused on understanding the underlying mechanisms that determine this polarization phenomenon from similar growing neurites, and major molecules such as Cdc42, PIP₃, GSK-3β and PAR-3 have been identified as playing a critical role [52-55]. The environmental signals that can effectively influence polarization have been studied to a lesser extent. Biochemical cues such as laminin [56] and NGF [57] increase the number of hippocampal cells in stage 3 (i.e., polarized cells) during the first 24 h in culture. Additionally, physical cues such as tension [58] and topographical features [59] also influence the directionality or rate of polarization. However, combination of contact guidance and immobilized growth factors has not been explored before for polarization and axon elongation in neurons.

Here we describe our efforts to investigate how surface properties influence the rate of polarization and axon length of hippocampal cells. In particular, we studied the combination of two different material surface properties: topographical characteristics that provide contact guidance and biochemical signals. To provide these two signals, microchannels of 1-2 µm in width were microfabricated on PDMS, a biocompatible, inexpensive and very common material in soft lithography applications, which allowed us to obtain multiple substrates by simple replica molding of the polymer. NGF was subsequently immobilized on these microstructures to create a bioactive surface. We found that the two stimuli could individually increase the percentage of neurons in stage 3 (i.e., initiation effect) after 20 h in culture with a more drastic effect for the topography, but there was no additive effect on initiation when both cues were present simultaneously. Additionally, these cues also increased axon length (i.e., elongation effect), with a more pronounced impact for the immobilized NGF and a synergistic increase for the simultaneous stimuli. We also found that microtopography also affected the orientation of axon growth by
producing larger axonal alignment when deeper and wider microchannels were present, which is consistent with previous reports.

5.2 Materials and Methods

5.2.1 Microfabrication of Channels

Microchannels 1 and 2 µm wide and 400 and 800 nm deep were created on PDMS using soft lithography techniques. The procedure consisted of three steps: 1) fabrication of a mask with the desired patterns, 2) fabrication of a silicon master, and 3) replica molding of the PDMS.

The mask was created with electron beam (E-beam) lithography. 4-µm-thick low temperature oxide was initially deposited on a silicon wafer (WaferWorld, West Palm Beach, FL) using low pressure chemical vapor deposition (LPCVD). The wafer was then annealed at 1000°C for 1 h to enhance film density and etching durability. The wafers were subsequently cut into 1 cm²-pieces and cleaned by sonication in acetone, isopropyl alcohol and water. 4% poly(methyl methacrylate) PMMA resist in chlorobenzene (Microchem, Newton, MA) was spin coated for 45 seconds and 3000 rpm, and baked for 30 minutes at 170°C. Microchannels of 1 or 2 µm were written on the PMMA resist using E-beam (Raith-50 and XL-30 SEM, LaB₆ source) with an area dose of 220 µAs/cm² and beam current of 0.2 nA. After the patterns were written, the exposed areas were developed with a mixture of methyl-isobutyl-ketone and isopropyl alcohol (1:3) (Sigma, St. Louis, MO) for 1.5 minutes.

After the microchannels were written with E-beam, this was followed by thermal evaporation (Denton) of a 100 nm chromium (R.D. Mathis, Long Beach, CA) film and lift-off with acetone, which created a Cr mask on the silicon substrate. Reactive Ion Etching (RIE) of the SiO₂ layer was performed in an etcher (Plasma Technology) with a mixture of CHF₃ and oxygen until the desired
thickness was obtained and measured with a Nanospec (Nanometrics). The Cr-coated areas were wet-etched with a mixture of ceric sulfate and nitric acid (Transene, Danvers, MA) for 2 minutes. The final master was silanized with tridecafluoro-1,1,2,2-(tetrahydrooctyl) trichlorosilane (Gelest, Morrisville, PA).

The microchannels on PDMS were produced by pouring a mixture of Sylgard 184 (Dow Corning, Midland, MI) on the master (one part curing agent per ten parts base), curing for 24 h at room temperature and releasing the film. This step was performed multiple times for a single master.

5.2.2 NGF-FITC Conjugation

NGF was conjugated to fluorescein for detection and characterization of the immobilization procedure. 40 µL of sodium bicarbonate buffer (0.1 M, pH = 9) was mixed with 100 µL of NGF 2.5S (Promega, Madison, WI, 100 µg/ml) and 10 µL of fluorescein isothiocyanate in dimethyl sulfoxide (FITC, 12 mg/mL) (Molecular Probes, Carlsbad, CA). The reaction was carried out at 4°C for 10 h. The unreacted FITC was separated by centrifugation using size exclusion chromatography columns (Biorad, Hercules, CA, exclusion limit 6,000 Da). Conjugation efficiency and degree of labeling were evaluated with a UV-Vis Beckman DU500 spectrophotometer by measuring absorbance at 280 nm and 494 nm. NGF-FITC was only used for quantification and visualization purposes, but not for cell culture because some loss of activity was detected in PC12 cell neurite extension assays (results not shown).

A calibration curve of NGF standards was obtained by casting known quantities of the fluorescent protein on defined areas without washing, calculating surface concentrations (ng/mm²) and capturing fluorescence images of the dry samples with a fluorescence microscope (IX-70, Olympus) using a constant exposure time. Before analysis, standards were exposed to UV light for 15 seconds (this was the exposure time for immobilizing NGF in the experiments), to
take into account any loss of fluorescence as a result of photobleaching. For all hippocampal cell culture, unlabeled NGF was used in the same quantities as used for NGF-FITC experiments.

5.2.3 NGF Immobilization

NGF photochemical fixation was performed using a phenyl-azido group, a method developed by Matsuda et al. [42] and modified by Ito and colleagues for immobilization of growth factors in particular [37,38,43]. The procedure consisted of three main steps (Figure 5.1): 1) preparation of N-4-(azidobenzoyloxy)succinimide according to a previously published procedure [42]; 2) polyallylamine (PAA) conjugation to N-4-(azidobenzoyloxy)succinimide, and 3) fixation of NGF using the modified polyallylamine. Briefly, N-4-(azidobenzoyloxy)succinimide was obtained by adding a solution of dicyclohexylcarbodiimide (Aldrich, St. Louis, MO) (6.7 g) in tetrahydrofuran (25 mL) to a solution of N-hydroxysuccinimide (Aldrich) (3.7 g) and 4-azidobenzoic acid (TCI America, Portland, OR) (4.8 g) in tetrahydrofuran (75 mL), followed by filtration and crystallization with isopropyl alcohol/diisopropyl ether (Aldrich) (see characterization in Appendix C). Subsequently, a solution of 15 mg of polyallylamine (Aldrich) in 10 mL of phosphate buffered solution (PBS, pH = 7.4) was added to a solution of 13 mg of N-4-(azidobenzoyloxy)succinimide in 5 mL of N,N-dimethylformamide and stirred for 24 h at 4°C. The solution was ultrafiltered (Millipore, Billerica, MA, 10,000 Da NMWL) and washed three more times by adding 10 mL of distilled-deionized (DDI) water and ultrafiltered again to finally obtain a volume of ~300 µL of photosensitive polyallylamine (PAA-Azido) (Appendix C).

The conjugate was analyzed by measuring UV-Vis absorbance of the filtration retentate at 280 nm. This conjugate was diluted in DDI water to obtain a final volume of 1.2 mL (1:4). 50 µL of this solution were cast on a PDMS
substrate (1 cm$^2$), air dried and exposed with a UV lamp (Blak-Ray, 22 mW/cm$^2$, $\lambda_{\text{max}} = 365$ nm) for 15 seconds followed by three washes with 0.05 M HCl and two washes with PBS. This step was followed by casting a second layer of the photosensitive polyallylamine (50 $\mu$L) and a superimposed final layer of NGF (for cell culture) or NGF-FITC (for quantification) (1-2 $\mu$g in 50 $\mu$L of PBS). For controls, PBS only was added instead of NGF solution, which produced a PDMS substrate only immobilized with PAA-azido. Finally, the substrate was exposed to UV light for 15 seconds and washed six times with PBS to remove unreacted protein and two more times with DDI water.

To prove the versatility of the immobilization procedure, patterning experiments were performed as proof of principle for other future applications (all cell culture experiments were performed with UV lamp immobilization). A TEM grid (Electron Microscopy Sciences) was placed on top of the dried layers before UV exposure to serve as a mask. Also, instead of using the UV lamp a solid state Nd:YAG laser (Surelite, Continuum) with a wavelength at 355 nm was used to create gradients of NGF on the surface by focusing and scanning lines with different exposure times. The substrate was placed on top of an automated stage, and the exposure time was controlled by the scanning rate. A laser energy of 6 $\mu$J was used with stage speeds between 20 and 180 $\mu$m/s.

5.2.4 NGF ELISA Assay

An ELISA assay was performed to check the concentration of any released NGF from the surface into the culture medium. PDMS substrates with immobilized NGF ($n=3$) were placed in 3 cm tissue culture dishes and incubated in PBS for 3 days at 37°C. Volumes of 200 $\mu$L were collected from the dishes at 6 h, 24 h, 48 h, and 72 h and analyzed with a commercially-available sandwich ELISA kit (NGF E$_{\text{max}}$ kit, Promega). Briefly, 96-well ELISA plates were coated with a primary goat anti-NGF antibody overnight at 4°C, followed by incubation
with blocking buffer from the kit (1 h) and incubations of samples and standards for 6 h at room temperature. Finally, a secondary rat anti-NGF antibody was incubated overnight, followed by incubation with anti-rat antibody conjugated to horseradish peroxidase (HRP) for 2.5 h, and development with 3,3',5,5'-tetramethylbenzidine (TMB). HCl 1 M was added to all wells, and absorbance at 450 nm was recorded using a plate reader.

5.2.5 Hippocampal Cell Culture

For cell culture, treated PDMS substrates were subsequently transferred to sterile 3 cm tissue culture dishes, washed twice with sterile water, air dried and stored at 4°C. Embryonic rat hippocampal cells (E18) were isolated from commercially-obtained hippocampus tissue (BrainBits, Springfield, IL). The hippocampi were incubated with papain (Worthington, Lakewood, NJ) in Hibernate E medium (Brainbits) (4 mg/mL) at 30°C for 20 minutes, followed by physical trituration with a fire-polished Pasteur pipette. Cells were counted and plated on already prepared PDMS substrates (7.5 x 10^3 cells/cm^2), and cultured with Neurobasal Medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen), L-glutamine (Fisher, 0.5 mM), L-glutamic acid (Sigma, 25 µM) and 1% antibiotic-antimycotic (Sigma, 10,000 units/mL of penicillin, 10 mg/mL of streptomycin and 25 µg/mL of amphotericin).

Cells were incubated on different substrates including PDMS with immobilized NGF and PDMS with PAA-azido and microchannels. Negative controls were PDMS with PAA-azido only. Positive controls included PDMS with PAA-azido and NGF in solution (10-50 ng/mL) and laminin-coated PDMS (incubation overnight at 4°C with 10 µg/mL). Laminin was used as a positive control because of its well-known effect on polarization [56].
5.2.6 Immunochemistry

After 20 h in culture at 37°C and 5% CO₂, cells were fixed with 4% paraformaldehyde (Sigma), 4% sucrose (Fisher) in PBS for 20 minutes, followed by permeabilization for 20 minutes with 0.1% Triton-X100 (Sigma) in 2% bovine serum albumin (BSA) (Jackson ImmunoResearch, West Grove, PA) in PBS, and blocking for 1 h at 37°C with 2% BSA-PBS. Samples were incubated with antibodies for Tau-1 (axonal marker) (Chemicon, Temecula, CA, 1:200) and NGF (Abcam, Cambridge, MA, 1:200) in 2% BSA-PBS overnight at 4°C, followed by incubation with fluorescently-labeled secondary antibodies (Alexa 488-conjugated, Molecular Probes, and TRITC-conjugated, Sigma), for 1 h at 37°C. All cell experiments were repeated at least three different times on different days.

5.2.7 Fluorescence Microscopy

NGF-FITC immobilization, cell polarization and axon extension were analyzed using an inverted phase contrast and fluorescence microscope. Images from the microscope were acquired using a color CCD video camera (Optronics MagnaFire, model S60800) and analyzed using Adobe Photoshop and Image J (NIH). For NGF coating analysis, fluorescence images of NGF-FITC-coated PDMS were captured with a constant exposure time and analyzed for intensity with the imaging software. For the different surface concentrations, experiments were repeated at least four separate times, and a total of 10-20 images were analyzed for fluorescence intensity per condition. Average of fluorescence intensity and standard error of the mean (SEM) are reported, and the average was compared to a calibration curve (see NGF-FITC conjugation section) to determine the surface concentration.
5.2.8 Polarization and Axon Length Analysis

Based on published criteria, a hippocampal cell was defined as polarized (stage 3) when one of its neurites was at least twice as long as the other neurites and it stained positively for Tau-1 \[50,52,53,55\]. The fraction of polarized cells was defined as the ratio of cells with axons to the total number of cells per sample. In the same experiments we also measured axon length and angle, which were defined as the straight-line distance from the tip of the axon to the junction between the cell body and axon base, and the angle between this line and a 0° line in the direction of the microchannels (see inset in Figure 5.7A). In the case of branched axons, the length of the longest branch was measured from the tip of the axon to the cell body, and then each branch was measured from the tip of the axon to the branch point. All experiments were repeated in duplicate at least three times on different days, and an average of 130 cells were analyzed per sample for each condition. P-values for fraction of polarized cells and axon length data were analyzed using 2-sided Student’s t-test with respect to controls as stated in Tables 5.1-5.4. Statistical significance was determined for P < 0.05.

5.2.9 Scanning Electron Microscopy (SEM)

Microchannels and cells on PDMS were analyzed with a LEO 1530 scanning electron microscope. To image microchannels, patterned PDMS substrates were coated with a gold layer a few angstroms thick and imaged with a typical acceleration voltage of 10 kV. To image neurons, cells on PDMS substrates were fixed with 4% paraformaldehyde (Aldrich) and 4% sucrose in PBS for 20 minutes, and dehydrated with increasing concentrations of ethanol (30% - 100%) for a total time of 2 h, followed by 5 minutes exposure to hexamethyl-disilazane (Sigma). After drying, samples were coated with a gold
layer for SEM measurement and imaged with a typical acceleration voltage of 1 kV.

5.2.10 Atomic Force Microscopy (AFM)

A Digital Instruments Dimension 3100 with Nanoscope IV controller was used in tapping mode to scan the PDMS substrates and measure microchannel depth.

5.3 Results and Discussion

5.3.1 Fabrication of Microchannels

Microchannels 1 and 2 µm wide and 400 and 800 nm deep were fabricated using conventional microlithographic techniques. These features were designed and etched on silicon wafers that served as masters for the replica molding of PDMS. Figure 5.2 illustrates representative SEM and AFM images for the 2 µm-wide and 400 nm-deep channels. Channel depths were also obtained for all other dimensions.

5.3.2 Immobilization of NGF

Photochemistry methods were utilized for tethering NGF to the surface of PDMS via a photosensitive intermediate layer composed of a backbone of polyallylamine with several pending phenylazido functional groups (PAA-azido) (Figure 5.1). This crosslinking compound was obtained from the acylation of polyallylamine by the reaction of amine groups from the polymer with N-4-(azidobenzoyloxy)succinimide, which incorporated the phenylazido moiety. UV-Vis spectra were used to confirm the effective incorporation of the photosensitive
group by measuring absorbance of the conjugate at 280 nm (results not shown). The final PAA-azido conjugate served as an intermediate linker for immobilizing NGF by activation of the phenylazido compounds with UV light and the subsequent nonspecific insertion into different bonds.

![Chemical Reaction Diagram]

**Figure 5.1.** Schematic of the NGF immobilization process on PDMS. PAA was conjugated to an azido compound to produce PAA-azido. This conjugate was cast twice on PDMS, followed by casting of NGF. UV light exposure promoted the formation of covalent bonds via the azido groups, immobilizing NGF to PDMS.
To analyze and detect the effectiveness of the immobilization, NGF was labeled with FITC, which was only used for detection purposes and not for cell culture. As observed in Figure 5.3, the chosen photochemistry permitted the immobilization of the protein in different schemes. In Figures 5.3A and 5.3B, NGF-FITC was homogeneously immobilized using a UV lamp, either on plain PDMS or over and within microchannels. This was the procedure used for all other experiments in this investigation. However, we also explored two more immobilization variations to show the versatility of the procedure. As shown in Figures 5.3C and 5.3D, the growth factor could be also selectively patterned by using either a mask during the exposure step with the UV lamp, or by using a precisely focused UV laser (Nd:YAG laser at 355 nm). A pattern from a TEM grid was successfully transferred to the surface of the PDMS using a UV lamp (Figure 5.3C), and a laser was used to create gradients by modulating the scanning rate of the laser (Figure 5.3D). These results successfully established the presence of the tethered protein on the surface of the material, which also indirectly confirmed the activation of the phenyl-azido groups with UV light, as NGF was only immobilized in the irradiated areas to create patterns.
NGF-FITC was also used to obtain an estimate of the surface concentration of the protein on PDMS. For this analysis, fluorescence intensity of the samples was compared with a calibration curve obtained by casting known quantities of NGF-FITC on defined areas. For the fluorescence intensity shown in Figure 5.3E, the approximate surface concentrations obtained when 1 or 2 µg of NGF were initially added corresponded to 0.08 and 0.11 ng/mm², respectively.
These values can be correlated with a 10% coverage when compared to the concentration of a typical protein monolayer (1 ng/mm\(^2\)) [70]. These values are similar to previous publications on immobilized NGF with concentrations of about 25 ng/cm\(^2\) [36]. However, it is important to clarify that probably not all immobilized NGF was biologically active.

Although we demonstrated the presence of the immobilized growth factor on the surface of PDMS, we also observed an important degree of nonspecific binding (without reaction) as illustrated in Figure 5.3E. The possible release into the media of this adsorbed NGF was analyzed with a time-course ELISA assay. This was important to consider to ensure that the observed effects on cells were caused by the immobilized and not the soluble form of NGF. Sample volumes were collected from PDMS-NGF substrates incubated in PBS at 37°C for 72 h (Figure 5.3F). The average concentration of NGF in solution was 1.34 ± 0.44 ng/mL (n = 3) which has negligible cellular effects [57]. Although soluble and immobilized concentrations cannot be directly compared, the soluble NGF concentration was probably much smaller than the immobilized NGF concentration (0.001 ng/mm\(^3\) compared to 0.11 ng/mm\(^2\)).

5.3.3 Polarization of Neurons on Microchannels and Immobilized NGF

Embryonic hippocampal cells in culture have been extensively studied because of their ability to spontaneously establish a single axon from equally growing neurites. These cells are the ideal model for analyzing axon initiation mechanisms, which involve accumulation of different markers in the neurite that becomes the axon [52-55]. We investigated how these cells polarized on a material with different surface properties such as topography provided by microchannels or bioactivity mediated by an active ligand. As observed in Tables 5.1 and 5.2 and Figures 5.4 and 5.5, there was a defined effect on the
fraction of stage 3 cells after 20 h in culture, depending on the surface characteristics.

Table 5.1. Fraction of Polarized Cells Cultured on Substrates with Single Cues

<table>
<thead>
<tr>
<th>Substrate Properties</th>
<th>Fraction of Polarized Cells*</th>
<th>P value (PAA-azido)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAA-azido (control)</td>
<td>0.22 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Microchannels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D)</td>
<td>0.37 ± 0.05</td>
<td>0.0042 *</td>
</tr>
<tr>
<td>1 µm(W), 0.4 µm(D)</td>
<td>0.31 ± 0.06</td>
<td>0.0802</td>
</tr>
<tr>
<td>2 µm(W), 0.8 µm(D)</td>
<td>0.39 ± 0.09</td>
<td>0.0158 *</td>
</tr>
<tr>
<td>1 µm(W), 0.8 µm(D)</td>
<td>0.32 ± 0.03</td>
<td>0.0274 *</td>
</tr>
<tr>
<td>Immobilized NGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>imm. NGF (1 µg)</td>
<td>0.25 ± 0.01</td>
<td>0.1846</td>
</tr>
<tr>
<td>imm. NGF (2 µg)</td>
<td>0.28 ± 0.02</td>
<td>0.0440 *</td>
</tr>
<tr>
<td>Ligand controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAA-azido + sol. NGF (10 ng/mL)</td>
<td>0.24 ± 0.03</td>
<td>0.6128</td>
</tr>
<tr>
<td>PAA-azido + sol. NGF (50 ng/mL)</td>
<td>0.31 ± 0.03</td>
<td>0.0348 *</td>
</tr>
<tr>
<td>Laminin (10 µg/mL)</td>
<td>0.29 ± 0.01</td>
<td>0.0400 *</td>
</tr>
</tbody>
</table>

*a Average Fraction ± SEM after 20 h in culture for <n> = 5  
(n = number of experimental samples)  
b 2-sided t-test compared to PAA-azido  
* Statistical difference for α = 0.05

Table 5.1 summarizes the results for individual stimuli presented on the surface of PDMS, which included physical cues (microchannels of 400 and 800 nm depth and 1 and 2 µm width) and biochemical cues (immobilized NGF or adsorbed laminin). These data suggest that the greatest effect for a single stimulus on the percentage of polarized cells was obtained by the presence of microchannels, more specifically of 2 µm width, in which there was an increase of 68 - 72% in the number of cells with an established axon compared to smooth substrates (i.e., PAA-azido only). The substrates with immobilized NGF also accelerated neuron polarization but to a lesser extent, with a 27% increase in stage 3 cells with respect to PAA-azido-only substrates. Although this effect was less pronounced than for topography, it was statistically different from PAA-azido controls (P = 0.04), and statistically indistinguishable from controls with either soluble NGF (50 ng/mL) (P = 0.46) or laminin coating (10 µg/mL) (P = 0.74).
Table 5.2. Fraction of Polarized Cells Cultured on Substrates with Combinatorial Cues

<table>
<thead>
<tr>
<th>Substrate Properties</th>
<th>Fraction of Polarized Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P value (PAA-azido)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P value (microchannels)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P value (NGF)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>600-nm Microchannels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D) + imm. NGF (1 µg)</td>
<td>0.32 ± 0.02</td>
<td>0.0160 *</td>
<td>0.3810</td>
<td>0.0284 *</td>
</tr>
<tr>
<td>1 µm(W), 0.4 µm(D) + imm. NGF (1 µg)</td>
<td>0.32 ± 0.03</td>
<td>0.0202 *</td>
<td>0.7952</td>
<td>0.0388 *</td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D) + imm. NGF (2 µg)</td>
<td>0.38 ± 0.02</td>
<td>0.0002 *</td>
<td>0.8572</td>
<td>0.0060 *</td>
</tr>
<tr>
<td>1 µm(W), 0.4 µm(D) + imm. NGF (2 µg)</td>
<td>0.31 ± 0.02</td>
<td>0.0132 *</td>
<td>0.9730</td>
<td>0.4224</td>
</tr>
<tr>
<td>1200-nm Microchannels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.8 µm(D) + imm. NGF (1 µg)</td>
<td>0.37 ± 0.03</td>
<td>0.0016 *</td>
<td>0.8324</td>
<td>0.0020 *</td>
</tr>
<tr>
<td>1 µm(W), 0.8 µm(D) + imm. NGF (1 µg)</td>
<td>0.35 ± 0.03</td>
<td>0.0042 *</td>
<td>0.5386</td>
<td>0.0072 *</td>
</tr>
<tr>
<td>2 µm(W), 0.8 µm(D) + imm. NGF (2 µg)</td>
<td>0.39 ± 0.02</td>
<td>6.5 x 10⁻⁵ *</td>
<td>0.9512</td>
<td>0.0026 *</td>
</tr>
<tr>
<td>1 µm(W), 0.8 µm(D) + imm. NGF (2 µg)</td>
<td>0.35 ± 0.02</td>
<td>0.0004 *</td>
<td>0.3590</td>
<td>0.0240 *</td>
</tr>
<tr>
<td>Microchannels + Soluble NGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D) + sol. NGF (50 ng/mL)</td>
<td>0.30 ± 0.09</td>
<td>0.1754</td>
<td>0.5080</td>
<td>0.9484</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average fraction ± SEM after 20 h in culture for <i>n</i> = 5

<sup>b</sup> 2-sided t-test compared to PAA-azido

<sup>c</sup> 2-sided t-test compared to the corresponding microchannel dimensions in the combination

<sup>d</sup> 2-sided t-test compared to the corresponding NGF concentration in the combination

* Statistical difference for α = 0.05
Figure 5.4. Contrasting effects of microchannels versus immobilized NGF on polarization and axon length. Representative data (complete data in Tables 5.1-5.4) of polarization and axon length of hippocampal cells cultured for 20 h on PDMS with different surface properties. A) Fraction of polarized hippocampal cells cultured on PDMS with different physical and chemical surface properties as stated in the X-axis. There was no additive effect from the combination of immobilized NGF and topography. *, ** Statistically significant differences from PDMS-PAA-azido and immobilized NGF alone, respectively, using t-test with P < 0.05; B) Axon length of hippocampal cells cultured on PDMS with different physical and chemical surface properties as stated in the X-axis. There was a synergistic effect from the combination of immobilized NGF and topography. *, **, *** Statistically significant differences from PDMS-PAA-azido, microchannels alone and immobilized NGF alone, respectively, using t-test with P < 0.05.
Figure 5.5. Phase-contrast and fluorescence photomicrographs of hippocampal cells on PDMS with individual stimuli. A-D) Cells cultured on PDMS-PAA-azido only; E-H) Cells cultured on 2 µm-wide and 400 nm-deep microchannels; I-L) Cells cultured on immobilized NGF (0.11 ng/mm²). Green (Alexa 488) and red (TRITC) labeling correspond to Tau-1 and NGF, respectively. Immobilized NGF on the surface was detected from substrate fluorescence, as shown in images J-L. Scale bar = 50 µm.
It is important to emphasize that these cellular responses were not the result of soluble NGF that had leached out from the surface; only 1 ng/mL of NGF was detected with ELISA assays and controls with 10 ng/mL soluble NGF did not elicit any significant polarization of hippocampal cells (see Table 5.1). In addition, it is also important to clarify that although hippocampal cells have intracellular NGF compartments, as seen from the NGF labeling in Figures 5.5 and 5.6, these cells only release NGF in very low quantities (~4-8 pg/mL for transfected cells with increased NGF expression) and as a consequence of high levels of calcium or glutamate for example [60].

Physical and biochemical stimuli were combined in a single modified material surface to investigate if the combination of these two simultaneous cues could further enhance axon initiation. In this case, microchannels were coated with immobilized NGF (as in Figure 5.3B) and cells observed for polarization. As summarized in Table 5.2 and Figure 5.4A, the combination strategy with 1 and 2 μm channels (400 nm deep) and immobilized NGF (both with 1 and 2 μg added) was statistically different from immobilized NGF only, but not statistically different from topography only, suggesting that contact guidance was the dominant stimulus. Similar behavior was observed with 800 nm-deep microchannels. The same result was also obtained when the cells were incubated on microchannels and NGF in solution. This suggests that although both soluble and immobilized NGF did increase the rate of polarization and the establishment of a single axon, these effects were attenuated in the presence of surface topography, which appears to be a dominant signal for polarization.

The effects of NGF in hippocampal cells have been controversial, as NGF seems to produce both an increase in the rate of polarization and apoptosis [57,61,62]. Nevertheless, Brann et al. proposed that such varied responses are correlated to neuron age [62]. This age effect is related to NGF intracellular
pathway through ceramide derived from sphingomyelin hydrolysis after binding to p75<sup>NTR</sup> receptor, as these cells do not express the high-affinity TrKA receptors. Although increased intracellular levels of ceramide do increase the number of cells in stage 3 during the first 24 h in culture, these ceramide levels also get much higher as the expression of p75<sup>NTR</sup> receptor increases with time and the production of ceramide increases accordingly, which ultimately ends in apoptosis.

With regard to enhanced neuron polarization, previous studies have demonstrated beneficial effects of soluble NGF with concentrations between 50 and 200 ng/mL in cultures with and without a supporting layer of glia [57]. An approximate increase of 32% in stage 3 neurons with 50 -100 ng/mL of soluble NGF without glia has been reported, which was analogous to the 36% increase observed in our control experiments with 50 ng/mL of NGF. Our results also suggest that these effects seem to be similar when the growth factor is presented in an immobilized form with an approximate surface concentration of 0.1 ng/mm<sup>2</sup> (see Table 5.1). Although p75<sup>NTR</sup> receptor produces clathrin-coated vesicles after NGF binding, this occurs at a much slower rate than for TrkA receptors [63], which suggests that intracellular cascades could be triggered at the cellular membrane, an advantage for applications with material-bound ligands. More importantly, signaling from this receptor does not require endocytosis as sphingomyelin hydrolysis (ceramide source) is not reduced when internalization is prevented [64]. This evidence supports the fact that immobilized NGF could have an effect in hippocampal cells similar to soluble NGF. Although tethered ligands cannot be internalized, the binding of immobilized NGF to P75<sup>NTR</sup> receptor could be sufficient to trigger ceramide production and therefore, accelerate polarization.
Figure 5.6. SEM, phase-contrast and fluorescence photomicrographs of hippocampal cells on PDMS with combinatorial stimuli. A,C,E) Cells cultured on 2 μm-wide and 800 nm-deep microchannels with immobilized NGF (0.11 ng/mm²); B,D,F) Cells cultured on 1 μm-wide and 400 nm-deep microchannels with immobilized NGF (0.11 ng/mm²). Fluorescence images correspond to the overlay of Tau-1 (green, Alexa 488) and NGF (red, TRITC) labeling. Neurons extended longer and more oriented axons on surfaces with combinatorial cues. Scale bars = 50 μm (A-D), 10 μm (E,F).

The effects of surface topography in polarization have been investigated to a lesser extent. Dowell-Mesfin et al. showed that pillars of 2 μm width increased the number of cells in stage 3 after 24 h in culture [59], but quantitative
measurements and comparison with chemical ligands have not been performed. More recently, Foley et al. showed increased neuritogenesis on nano-ridges in PC12 cells with low levels of NGF [68], and Ahmed et al. reported an increase in neurite generation in several neuronal types cultured on nanofibers [9]. We confirmed that microtopography increases the number of cells in stage 3 after 20 h in culture, with a 68% and 72% increase for 2 µm microchannels (400 and 800 nm, respectively) and a 36% and 42% increase for 1 µm microchannels (400 and 800 nm, respectively) with respect to PAA-azido-only (smooth) substrates (Table 5.1). Furthermore, our results suggest that the effect of topography on the rate of polarization was greater than other controls such as NGF in solution or laminin.

Intracellular signaling derived from contact guidance cues is less well-understood than growth-factor signaling. These responses are thought to be transduced by tension generated within the cytoskeleton and the redistribution of focal adhesion complexes (FAC) caused by surface topography [reviewed in 65]. In particular, integrins in FAC are important modulators of intracellular signaling, and additionally, the cytoskeleton is directly connected to the nuclear membrane, which ultimately alters nucleus morphology in the presence of topography. It has been hypothesized that nuclear alignment could be connected to changes in gene expression [65]; for example it was shown by Dalby et al. that numerous genes were upregulated in fibroblasts when nuclei were aligned with respect to topographical patterns [66]. Based on this, polarization in hippocampal cells could be highly influenced by topographical features as a consequence of signal transduction by the cytoskeleton to the nucleus, and subsequent effects on protein translation. We speculate, given the results analyzed here, that signal transduction through the cytoskeleton could be more effective in triggering polarization compared to signaling from NGF.
5.3.4 Axon Length and Orientation of Hippocampal Cells

In addition to analyzing the fraction of stage 3 cells, we also analyzed the length and orientation of the already established axons, and, interestingly, the results were not analogous. Immobilized NGF alone elicited an increase of 10% in axon length with respect to PAA-azido only samples, whereas topography did not significantly affect axon length, except for microchannels of 1 µm width and 800 nm depth, which had an increase of 20% (Table 5.3). Although we expected an increase in axon initiation to be correlated with longer axons, the results revealed that topography had little effect on elongation rate after polarization. In contrast, neurons exposed to immobilized NGF had initially slower polarization, but the growth rate must have been enhanced after initiation to ultimately produce longer axons. NGF in solution (50 ng/mL) and laminin also appeared to have more pronounced effects on axon length than on axon initiation, as these ligands produced an increase in length of 10% and 20%, respectively, compared with PAA-azido-only substrates. Again, the similarity in responses between immobilized NGF and soluble NGF, exhibiting the same increase in length, further supports the possibility of triggering equivalent intracellular cascades.

Quantitative analysis of the effects of soluble NGF in axon length in hippocampal cells has not been reported before. However, Schwarz and Futerman [67] investigated the effect of adding ceramide stereoisomers to hippocampal cell cultures, which would mimic the effect of NGF, as ceramide is critical in the intracellular pathway of NGF as discussed before. In that study, an increase of 40% in length was detected with large quantities of ceramide (5 µM), although this cannot be directly correlated to a particular amount of NGF. However, the authors reported that a significant increase in the number of polarized cells was achievable with much lower concentrations of ceramide (0.05-0.1 µM), which suggested that low concentrations of ceramide might be
effective in enhancing polarization, but not axon growth extensively. In other
words, ceramide produced from NGF binding to p75NTR would more effectively
impact polarization than axon elongation, although the overall effect on
polarization is small when compared to topography, and the effect on growth is
significant when compared to topography as well. Here we observed a rather
small increase of 10% in length for both soluble and immobilized forms of NGF,
although the difference was statistically different from controls with PAA-azido-
only substrates (P < 0.03).

Table 5.3. Axon Length of Cells Cultured on Substrates with Single Cues

<table>
<thead>
<tr>
<th>Substrate Properties</th>
<th>Average length (µm)a</th>
<th>P value (PAA-azido) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAA-azido (control)</td>
<td>61.01 ± 1.69</td>
<td></td>
</tr>
<tr>
<td>Microchannels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D)</td>
<td>57.16 ± 3.87</td>
<td>0.3188</td>
</tr>
<tr>
<td>1 µm(W), 0.4 µm(D)</td>
<td>64.51 ± 3.63</td>
<td>0.3906</td>
</tr>
<tr>
<td>2 µm(W), 0.8 µm(D)</td>
<td>63.62 ± 3.65</td>
<td>0.4876</td>
</tr>
<tr>
<td>1 µm(W), 0.8 µm(D)</td>
<td>73.01 ± 3.73</td>
<td>0.0012 *</td>
</tr>
<tr>
<td>Immobilized NGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>imm. NGF (1 µg)</td>
<td>68.05 ± 1.49</td>
<td>0.0018 *</td>
</tr>
<tr>
<td>imm. NGF (2 µg)</td>
<td>65.77 ± 1.19</td>
<td>0.0258 *</td>
</tr>
<tr>
<td>Ligand Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAA-azido + sol. NGF (10 ng/mL)</td>
<td>61.23 ± 2.86</td>
<td>0.9522</td>
</tr>
<tr>
<td>PAA-azido + sol. NGF (50 ng/mL)</td>
<td>68.77 ± 2.61</td>
<td>0.0118 *</td>
</tr>
<tr>
<td>Laminin (10 µg/mL)</td>
<td>73.74 ± 2.79</td>
<td>7.64 x 10⁻⁵ *</td>
</tr>
</tbody>
</table>

a Average length ± SEM after 20 h in culture for <n> = 139
(n = number of analyzed axons)
b 2-sided t-test compared to PAA-azido
* Statistical difference for α = 0.05

In addition to polarization, we were interested in combining microchannels
and immobilized NGF to detect any cooperative enhancement in axon length. As
observed in Table 5.4, there seemed to be an effect from the combination of
stimuli as surfaces with both cues had a maximum increase of 21% and 25% in
length for 2 µm and 1 µm microchannels (400 nm depth), respectively, compared
with PAA-azido-only samples. These percentages were significantly different from the values for individual stimuli (see P-values in Table 5.4), and because topography alone did not elicit an increase in neurite length, a synergy mechanism must have been present. There was a similar trend for 800 nm-deep and 2 µm-width microchannels, with an increase of 28% in length compared to PAA-azido, but there was not a significant combinatorial effect for the 1 µm channels, which already had a large effect when the topography was presented individually (20%). The observed synergy might be a product of faster polarization on topography in conjunction with the enhanced growth rate from NGF, which might ultimately yield longer axons. This suggests that to have the longest neurites, it is necessary to combine fast initiation with fast growth, which in this study was obtained by combining bioactive plus topographical properties on the material surface.

In relation to axon orientation, we observed results similar to those published for hippocampal cells in the literature [18,69] (Figures 5.6 and 5.7). Figures 5.6 and 5.7A illustrate that cells tended to grow perpendicular (angle = 90°) to the 1 µm microchannels (400 nm deep), and to some extent also in the 2 µm channels, although for this width cells also grew in a parallel fashion (angle = 0° or 180°). However, as depth increased to 800 nm the percentage of cells growing parallel to the microchannels increased, which was more dramatically observed for the 2 µm channels (Figures 5.6 and 5.7B).
Table 5.4. Axon Length of Cells Cultured on Substrates with Combinatorial Cues

<table>
<thead>
<tr>
<th>Substrate Properties</th>
<th>Average length (µm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P value (PAA-azido)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P value (microchannels)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P value (NGF)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>600-nm Microchannels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D) + imm. NGF (1 µg)</td>
<td>74.16 ± 3.12</td>
<td>0.0002 *</td>
<td>0.0006 *</td>
<td>0.0680</td>
</tr>
<tr>
<td>1 µm(W), 0.4 µm(D) + imm. NGF (1 µg)</td>
<td>70.83 ± 3.69</td>
<td>0.0084 *</td>
<td>0.2396</td>
<td>0.4288</td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D) + imm. NGF (2 µg)</td>
<td>68.13 ± 3.47</td>
<td>0.0396 *</td>
<td>0.0416 *</td>
<td>0.4600</td>
</tr>
<tr>
<td>1 µm(W), 0.4 µm(D) + imm. NGF (2 µg)</td>
<td>76.17 ± 3.30</td>
<td>7.58 x 10&lt;sup&gt;-6&lt;/sup&gt; *</td>
<td>0.0356 *</td>
<td>0.0006 *</td>
</tr>
<tr>
<td><strong>1200-nm Microchannels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.8 µm(D) + imm. NGF (1 µg)</td>
<td>74.67 ± 4.40</td>
<td>0.0008 *</td>
<td>0.0538</td>
<td>0.0826</td>
</tr>
<tr>
<td>1 µm(W), 0.8 µm(D) + imm. NGF (1 µg)</td>
<td>70.93 ± 3.43</td>
<td>0.0074 *</td>
<td>0.6832</td>
<td>0.4114</td>
</tr>
<tr>
<td>2 µm(W), 0.8 µm(D) + imm. NGF (2 µg)</td>
<td>78.32 ± 3.09</td>
<td>1.38 x 10&lt;sup&gt;-7&lt;/sup&gt; *</td>
<td>0.0048 *</td>
<td>1.02 x 10&lt;sup&gt;-5&lt;/sup&gt; *</td>
</tr>
<tr>
<td>1 µm(W), 0.8 µm(D) + imm. NGF (2 µg)</td>
<td>68.81 ± 2.17</td>
<td>0.0044 *</td>
<td>0.3062</td>
<td>0.2248</td>
</tr>
<tr>
<td><strong>Microchannels + Soluble NGF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µm(W), 0.4 µm(D) + sol. NGF (50 ng/mL)</td>
<td>74.85 ± 6.63</td>
<td>0.0037 *</td>
<td>0.0214 *</td>
<td>0.3156</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average length ± SEM after 20 h in culture for <i>n</i> = 139 (n = number of analyzed axons)
<sup>b</sup> 2-sided t-test compared to PAA-azido
<sup>c</sup> 2-sided t-test compared to the corresponding microchannel dimensions in the combination
<sup>d</sup> 2-sided t-test compared to the corresponding NGF concentration in the combination

* Statistical difference for α = 0.05
Figure 5.7. Axon orientation of hippocampal cells after 20 h in culture on PDMS with different surface properties. A) Angle distribution for axons growing on 400 nm-deep microchannels with 0.11 ng/mm² of immobilized NGF (n = 287, 137, 122, for PAA-azido, 1 μm and 2 μm, respectively). Inset: schematic illustrating how axon length and angle were analyzed; B) Angle distribution for axons growing on 800 nm-deep microchannels with 0.11 ng/mm² of immobilized NGF (n = 287, 198, 170, for PAA-azido, 1 μm and 2 μm, respectively). Axons tended to grow parallel to microchannels as depth and width increased.

Effects of topography on neurite length and orientation have been reported previously. Rajnicek et al. reported that smaller widths produce longer neurites [18], which is also suggested in Table 5.3, although we only saw significant differences from the control for the 1 μm-wide and 800 nm-deep channels. Perpendicular and parallel growth to microchannels was also thoroughly investigated by the same group who reported similar trends as observed here, with increasing parallel orientation with increasing widths and depths, and the
authors also analyzed the important role of calcium influx and protein kinase C in this behavior [69]. More recently, Dowell-Mesfin et al. reported that after 14 days of culture, hippocampal cells had longer neurites for smaller distances between ridges, neurites tended to bridge in between adjacent pillars [59], which can also be observed in Figure 5.6F. This bridging is what finally allows the neurites to grow in a perpendicular fashion, which becomes more difficult as groove width increases. These authors also reported changes in growth cone morphology depending on the gap size between pillars, with growth cones having more “narrow profiles” for the smaller gaps. Based on this observation, we speculate that 1 µm-wide and 800 nm-deep microchannels could have the largest effect on neurite length because the growth cone is more restricted in movement and morphology. Similarly, the impact of smaller patterns on axon length could have also played a role in the synergy observed for 1 µm microchannels (400 depth) and immobilized NGF.

5.4 Conclusions

We have investigated hippocampal cell responses to different material surface properties, including microtopography and immobilized NGF. We found that immobilized NGF (0.1 ng/mm²) produced similar responses as soluble NGF (50 ng/mL), which allowed us to hypothesize the equivalence of intracellular pathways, even without endocytosis in the immobilized form. Neuron polarization was analyzed and we found that surface topography influenced more effectively the fraction of stage 3 neurons (68% compared to controls) than biochemical ligands such as NGF or laminin (30% increase). Furthermore, when both types of cues were simultaneously presented on the surface of PDMS (i.e., microchannels plus tethered NGF) there was only a significant increase with respect to immobilized NGF but not to topography itself, suggesting that physical signals have a dominant effect on polarization mechanisms.
In contrast, effects on axon length seemed to be opposite. Immobilized NGF had a greater but modest increase (10% increase with respect to controls), whereas topography did not have a significant effect (except for 1 μm-width and 800 nm-depth). However, a synergistic enhancement was observed when both contact guidance and growth factor were presented simultaneously on the surface (25% increase), which could be a result of faster polarization from topography plus increased growth rate from NGF. Finally, axon orientation was also investigated. Our data were consistent with previous results, with more parallel alignment of axons with larger widths and depths for microchannels.

These studies not only compared responses between chemical and physical stimuli, but also investigated how these cues are modulated when presented simultaneously on the surface of a material. Axon formation (initiation) and axon growth (maintenance) were independently studied, and our results suggest a more prominent effect of topography on initiation, in contrast to the more effective role of immobilized NGF in axon growth after initiation. These data imply the need to combine fast initiation with fast growth to ultimately achieve longer axons, which was accomplished here by combining biochemical and physical properties on the surface of the material. From this it is evident that surface characteristics can be precisely engineered to present combinatorial cues that target multiple cellular transduction mechanisms to finally produce enhanced neuronal responses. These results can be potentially applied to the design of biomaterials used to interface with neurons.

5.5 References


[16] Saneinejad S, Shoichet MS. Patterned glass surfaces direct cell adhesion and


Chapter 6
Combinatorial Strategy II: Polypyrrole and Immobilized NGF

Introduction

Biomaterials that present multiple stimuli are attractive for a number of biomedical applications. In particular, electrical and biological cues are important factors to include in interfaces with neurons for applications such as nerve conduits and neural probes. This chapter describes the creation of a second novel combinatorial strategy, where polypyrrole surface is modified with immobilized NGF to provide both electrical and chemical stimulation to neurons. The immobilization procedure is similar to the one explained in Chapter 5, but using polypyrrole instead of PDMS. PC12 cells were used for neurite extension analysis, as these cells were previously used in electrical stimulation experiments in the literature.

6.1 Background and Motivation

Neurons are highly influenced by electrical stimuli because of their inherent nature in transmitting electrochemical signals throughout the nervous system. As a consequence, engineered materials and devices capable of recording or stimulating nerve cells with electrical mechanisms have been investigated extensively. In addition to electrical stimulation, it is desirable to create materials that are biomimetic and thus integrate other cues that could enhance neuronal interfacing by inducing cell adhesion, recognition, neurite extension and reducing inflammation and scar tissue. For example, microfabricated neural probes have been modified to present or deliver biomacromolecules that promote a more intimate contact with the surrounding
tissue, improve recording performance, or chemically stimulate neurons [1-3]. Silicon probes have been fabricated with hollow microchannels that can locally deliver compounds such as the neuromodulator γ-aminobutyric acid (GABA) [1] and therapeutic drugs to reduce inflammatory signals [2]. Polyimide electrodes have also been modified to release NGF via dextran hydrogels [3]. These electrodes were reported to produce functional interfaces by supporting neurite growth toward the recording sites.

Polypyrrole (PPy), a biocompatible electrically-conductive polymer, has been used for neural prosthetics [4-9], in addition to *in vitro* and *in vivo* studies for nerve regeneration [10-14] and other tissue engineering applications [15-20]. PPy is an attractive material for neural probes because of its biocompatibility, good conductivity, ease of synthesis and the potential to have high surface areas [9]. Similarly, PPy has been investigated as a nerve conduit material because of its electrical characteristics and biocompatibility [12,14]. However, it is still desired for these and other applications to provide additional cues such as biomolecules in conjunction with electroactivity provided by the conducting polymer. The ideal properties of nerve conduits for nerve regeneration are to include electrical stimulation with growth factors, biodegradability and topographical features [21]. Likewise, PPy coatings on electrodes and other devices have been combined with biomolecules to enhance cell interfacing. Adhesive peptides such as RGD and YIGSR [5,8,17], polysaccharides such as heparin, hyaluronic acid and dextran [15,19,20,22] and proteins such as human serum albumin and NGF [22] have been incorporated into PPy as dopants. However, doping of biomolecules has some limitations such as low loading, decrease in conductivity (~ 3 - 4 orders of magnitude difference [20]), and in the case of induced release upon reduction of the polymer, the supply is limited and the release is rather fast [22]. As an alternative, surface immobilization of macromolecules has been explored to overcome these limitations. For example,
PPy has been functionalized with hyaluronic acid by grafting amino-silanes on the surface [16,23], and other biomolecules such as heparin and enzymes have also been immobilized [24,25]. RGD sequences have been grafted via either cystine residues [17,26], or peptides that selectively bind to PPy [27]. More recently, immobilization has been performed via reactions with dopants that introduce reactive functional groups (e.g., glutamate) [28], but could decrease conductivity. Although these techniques do not drastically affect PPy properties, some of them are complex and have not been used for tethering growth factors.

NGF is the most studied and characterized neurotrophin, known for inducing several neuron responses, including neurite outgrowth [29-31]. This process is not necessarily mediated by retrograde transport [32-34] and is thought to be controlled by localized actin polymerization [35]. As a consequence, NGF immobilized to different surfaces has proven to be effective in inducing neurite extension, turning and sprouting [33,34,36-40]. Other growth factors have also been immobilized on substrates with retained activity [41-43]. A common approach for this protein immobilization is the use of arylazido-containing compounds [41,42,44-49] as explained in Chapter 5. Here we extended this approach for the surface decoration of PPy with NGF. This method is rather simple and in addition to the immobilization of the protein, the cell adhesive polymer polyallylamine (PAA) is also incorporated onto the surface of PPy, which further improves cell interfacing.

In an effort to modify PPy with a growth factor for chemical stimulation, we report the surface tethering of NGF mediated by a nonspecific arylazido-containing photolinker. This process rendered a conducting polymer capable of also stimulating neurons with specific ligands that trigger intracellular cascades to promote neurite extension. In addition, the conductivity of NGF-immobilized PPy (PPy-NGF) was not significantly affected, which was comparable to other surface
modification methods [16,23,26] and improved over the dopant method, and indicated it as a viable option to effectively combine multiple stimuli into a single material. Neurite outgrowth in PC12 cells cultured on this modified biomaterial (NGF surface concentration 1 ng/mm²) was possible without NGF added to the culture medium. Furthermore, electrical stimulation of cells using PPy-NGF revealed an increase in neurite length of 50% with respect to unstimulated controls. This modification of polypyrrole allows combining electrical stimulation from the polymer with biochemical ligands on the surface, which could synergistically stimulate cells. As a consequence, this approach represents an alternative for simultaneous chemical and electrical interfacing with cells and with only small alterations of the polymer properties.

6.2 Materials and Methods

6.2.1 Polypyrrole Synthesis

Polypyrrole (PPy) films were synthesized electrochemically as previously described [10,13]. Briefly, microscope glass slides were coated with thin layers of chromium (3 nm) and gold (30 nm) deposited with a thermal evaporator (Denton). These slides were used for the galvanostatic deposition of the conducting polymer. Specifically, a three-electrode setup was used, which consisted of the gold-coated slide as the working electrode, a platinum gauze counter electrode and a saturated calomel reference electrode. The films were deposited from an aqueous solution of 0.1 M pyrrole monomer (Aldrich) and 0.1 M sodium salt of poly(styrene sulfonate) (PSS) (Aldrich), using an offset voltage of 720 mV and a constant current of 7.2 mA. A Pine Instruments AFRDE5 bipotentiostat was used as the DC voltage source. Film thickness was determined by integrating current over time, and controlled by the passage of charge. Optically transparent films of 200 nm thickness were synthesized for cell
experiments, dried and desiccated. Thick films of 1.3 μm thickness were only synthesized for conductivity measurements. For NGF immobilization and neurite outgrowth (without electrical stimulation), PPy slides were cut into smaller pieces (1 cm²) with a diamond cutter. Complete slides were used for electrical stimulation of cells. Plexiglas chambers (1 cm X 1.5 cm) were attached to the slides and NGF immobilization and cell seeding were performed on the area enclosed by the chambers.

6.2.2 Conductivity Measurements

Conductivity measurements of PPy and PPy-NGF films were performed using a four-point probe technique [50]. PPy films were electrochemically synthesized to a thickness of 1.3 μm. The films were peeled off the gold-coated slides and fixed to unmodified glass slides with vacuum grease, and NGF immobilization was performed subsequently. A constant current was applied between two adjacent corners, and the voltage across the remaining two corners was measured to determine resistance. The conductivities of the films were calculated from these resistance measurements using the equations derived by Van der Pauw [50]. Experiments were repeated two times on separate days.

6.2.3 NGF-FITC Conjugation

NGF was conjugated to fluorescein for detection and characterization of the immobilization procedure. 40 μL of sodium bicarbonate buffer (0.1 M, pH = 9) was mixed with 100 μL of NGF 2.5S (Invitrogen, 100 μg/ml) and 10 μL of fluorescein isothiocyanate in dimethyl sulfoxide (FITC, 12 mg/mL) (Molecular Probes). The reaction was performed at 4°C for ~10 h. The unreacted FITC was separated by centrifugation with size exclusion chromatography columns (Biorad, exclusion limit 6,000 Da). Conjugation efficiency and degree of labeling were
evaluated with a UV-VIS Beckman DU500 spectrophotometer by measuring absorbance at 280 nm and 494 nm. NGF-FITC was only used for quantification and visualization purposes, but not for cell culture because some loss of activity was detected in PC12 cell neurite extension assays (results not shown).

A calibration curve for NGF characterization was obtained by casting known quantities of the fluorescent protein on defined areas without washing, calculating surface concentrations (ng/mm²) and capturing fluorescence images of the dry samples with a fluorescence microscope (IX-70, Olympus) using a constant exposure time. Before analysis, standards were exposed to UV light for 15 seconds (this was the exposure time for immobilizing NGF in the experiments), to take into account any loss of fluorescence as a results of photobleaching. For all cell culture, regular NGF was used in the same quantities as used as for NGF-FITC experiments.

6.2.4 NGF Immobilization

NGF photochemical fixation was performed using a phenyl-azido group, a method developed by Matsuda et al. [46] and modified by Ito and colleagues for immobilization of growth factors in particular [41,42,47]. The procedure consisted of three main steps (Figure 6.1): 1) preparation of N-4-(azidobenzoyloxy)succinimide according to a previously published procedure [46]; 2) polyallylamine (PAA) conjugation to N-4-(azidobenzoyloxy)succinimide, and 3) fixation of NGF using the modified polyallylamine. Briefly, N-4-(azidobenzoyloxy)succinimide was obtained by adding a solution of dicyclohexylcarbodiimide (Aldrich) (6.7 g) in tetrahydrofuran (25 mL) to a solution of N-hydroxysuccinimide (Aldrich) (3.7 g) and 4-azidobenzoic acid (TCI America) (4.8 g) in tetrahydrofuran (75 mL), followed by filtration and crystallization with isopropyl alcohol/diisopropyl ether (Aldrich) (characterization in Appendix C). Subsequently, a solution of 15 mg of polyallylamine (Aldrich) in 10 mL of
phosphate buffered solution (PBS, pH = 7.4) was added to a solution of 13 mg of N-4-(azidobenzoyloxy)succinimide in 5 mL of N,N-dimethylformamide and stirred for 24 h at 4°C. The solution was ultrafiltered (Millipore, 10,000 Da NMWL) and washed three more times by adding 10 mL of distilled-dionized water and ultrafiltered again to finally obtain a volume of ~300 µL of photosensitive polyallylamine (PAA-azido) (Appendix C).

The conjugation was confirmed by measuring UV-VIS absorbance of the filtration retentate at 280 nm. 50 µL of this solution were cast on a PPy substrate (1 cm²), air dried and exposed with a UV lamp (Blak-Ray, 22 mW/cm², λ_{max} = 365 nm) for 15 seconds followed by three washes with 0.05 M HCl and two washes with PBS. This step was followed by casting a second layer of the photosensitive polyallylamine (50 µL) and a superimposed final layer of NGF (for cell culture) or NGF-FITC (for quantification) (0.5-2 µg in 50 µL of PBS). For controls, PBS only was added instead of NGF solution, which produced a PPy substrate only immobilized with PAA-azido (PPy-PAA-azido). Finally, the substrate was exposed to UV light for 15 seconds and washed six times with PBS to remove unreacted protein. For neurite growth analysis, the treated substrates were subsequently glued with silicone to the bottom of sterile 3 cm-tissue-culture dishes. For electrical stimulation experiments, NGF was immobilized on a PPy area (1 cm²) enclosed by a Plexiglas chamber attached to the PPy slide.

6.2.5 NGF ELISA Assay

An ELISA assay was performed to check the concentration of any released NGF from the surface into the culture medium. PPy-NGF substrates (n = 3) were placed in 3 cm tissue culture dishes and incubated in PBS for 3 days at 37°C. Similarly, PPy-NGF slides with chambers for electrical stimulation were incubated in the same conditions. Volumes of 200 µL were collected from the
dishes at 6 h, 24 h, 48 h, and 72 h and analyzed with a commercially-available sandwich ELISA kit (NGF E\textsubscript{max} kit, Promega). Briefly, 96-well ELISA plates were coated with a primary goat anti-NGF antibody overnight at 4°C, followed by blocking and incubations of samples and standards for 6 h at room temperature. Finally, a second rat anti-NGF antibody was incubated overnight, followed by incubation with anti-rat antibody conjugated to horseradish peroxidase (HRP) for 2.5 h, and development with 3,3’,5,5’-tetramethylbenzidine (TMB). HCl 1 M was added to all wells, and absorbance at 450 nm was recorded using a plate reader.

6.2.6 X-ray Photoelectron Spectroscopy (XPS)

To confirm NGF immobilization, samples were characterized by XPS. Samples with and without immobilized NGF were analyzed. Spectra were taken on a PHI 5700 ESCA system using a monochromatic Al X-ray source operated at pass energies of 117.4 eV for surveys and 11.7 eV for high-resolution scans. The binding energy was calibrated using Au4f, Cu2p and Ag3d. Deconvolution peaks were obtained using an XPS peak fitting program (XPSPEAK 4, The Chinese University of Hong Kong) maintaining approximately constant the full width at half maximum (FWHF) for all peaks. A quantitative analysis of the N 1s/C 1s ratio was performed by calculating the ratio of the corresponding peak areas corrected by sensitivity factors, which results in the measurement of atomic ratios.

6.2.7 Cell Culture

PC12 cells (ATCC) were cultured in Ham’s F12K medium (Sigma) supplemented with 15% heat-inactivated horse serum (Hyclone), 2.5% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Sigma). For experiments with serum-free conditions, cells were cultured in Ham’s F12K medium with N-2 supplement (Invitrogen). Cells were cultured in standard tissue culture dishes and passaged 1:8 every 7 days. For neurite outgrowth experiments, 50 ng/mL
2.5S NGF was added to the culture medium when feeding cells for 1 week prior to the experiment (priming). NGF priming allows cells to grow neurites faster after plating. Cells were detached from the substrate using 0.25% Trypsin-EDTA (Sigma) and plated in 3 cm-tissue-culture dishes containing PPy substrates (1.25 x 10^4 cells/cm²) and analyzed for neurite extension after 2 or 10 days in culture. This last time-point was found to be appropriate for demonstrating the overall ability of tethered NGF to produce long neurites after several days in culture. Control samples were cultured with NGF in solution (50 ng/mL) whereas experimental PPy-NGF samples were cultured without NGF in the medium. Experiments were repeated four times on separate days.

After 2 or 10 days in culture at 37°C and 5% CO₂, PC12 cells were fixed with 4% paraformaldehyde (Sigma), 4% sucrose (Fisher) in PBS for 20 minutes, followed by permeabilization for 5 minutes with 0.1% Triton-X100 (Sigma) in 2% bovine serum albumin (BSA) (Jackson ImmunoResearch) in PBS, and blocking for 1 h with 2% BSA-PBS. Samples were incubated with phalloidin-TRITC (Sigma) in PBS for 30 minutes, washed and imaged.

Dorsal root ganglion (DRG) neurons were obtained from 2 week old Sprague-Dawley rat pups. The animals were acquired from a closed colony at the University of Texas Animal Resource Center and treated in accordance with the regulations established by the National Research Council in the Guide for the Care and Use of Laboratory Animals. Pups were euthanized with IsoFlo (Abbott Laboratories). The spinal column was then removed and the spinal cord exposed. The DRGs were then explanted into RPMI 1640 medium. Approximately 30 DRGs were collected from each animal, followed by incubation in Trypsin-EDTA for 30 minutes at 37°C, and incubation in collagenase in Neurobasal medium (Sigma, 200 ng/mL) for 30 minutes at 37°C. DRGs were triturated extensively with a fire-polished pipette for dispersing individual cells, followed by centrifugation and plating. Neurons were cultured with Neurobasal Medium (Invitrogen) supplemented with B-27 (Invitrogen), L-glutamine (Fisher,
0.5 mM), and 1% antibiotic-antimycotic (Sigma, 10,000 units/mL of penicillin, 10 mg/mL of streptomycin and 25 µg/mL of amphotericin), at a density of ~ 3 x 10³ cells/cm² (~ half DRG per substrate). After two days in culture, neurons were fixed, permeabilized and incubated with anti-GAP-43 (Sigma, 1:200) overnight, followed by incubation with a fluorescently-labeled secondary antibody (Alexa 488-conjugated, Molecular Probes). Experiments were repeated three times for two different animals.

6.2.8 Electrical Stimulation

Plexiglas chambers were attached to PPy slides for electrical stimulation and NGF was immobilized in the area enclosed by the chamber. Electrical stimulation conditions and priming time were exactly followed as previously reported [10] to compare neurite lengths with results in the literature. PC12 cells were primed for 24 h with soluble NGF (50 ng/mL) and subsequently seeded inside the Plexiglas wells (1.25 - 2 x 10⁴ cells/cm²). Cells were allowed to incubate for an additional 24 h before being electrically stimulated. The PPy film served as the anode, a gold wire attached to the Plexiglas chamber served as the cathode, and a silver wire served as a quasi-reference electrode. A steady potential of 100 mV was applied for a total time of 2 h per sample. Cells were maintained in a CO₂ incubator during the stimulation, and neurite lengths were analyzed 24 h after the stimulation. Experiments were run in duplicate and repeated three times on separate days.

6.2.9 Fluorescence Microscopy and Neurite Extension Analysis

NGF-FITC immobilization and neurite extension were analyzed using an inverted phase contrast and fluorescence microscope. Images from the
microscope were acquired using a color CCD video camera (Optronics MagnaFire, model S60800) and analyzed using Adobe Photoshop and Image J (NIH). Fluorescence images of NGF-FITC on PPy were captured with a constant exposure time and analyzed for intensity with the imaging software. For the different surface concentrations, experiments were repeated at least four separate times, and a total of 10-20 images were analyzed for fluorescence intensity per condition, averaged and the standard error of the mean (SEM) calculated. The average was compared to an established standard curve, as explained in the NGF-FITC conjugation section, to determine the surface concentration. The lengths of individual neurites were measured as previously described [10,13]. Length was defined as the distance from the tip of the neurite to the junction between the cell body and neurite base. In the case of branched neurites, the length of the longest branch was measured from the tip of the neurite to the cell body, and then each branch was measured from the tip of the neurite to the neurite branch point. Neurite length was not normally distributed; therefore the complete distribution and median length are reported. Statistical differences between medians were calculated with the nonparametric Kruskal-Wallis test [51] using the chi-square distribution for the level of significance.

6.2.10 Scanning Electron Microscopy (SEM)

PPy and PPy-NGF were analyzed with SEM for surface morphology. Substrates were covered with a gold layer of a few angstroms thick. A LEO 1530 scanning electron microscope was used with an acceleration voltage of 10 kV. Cells on PPy substrates were fixed with 4% paraformaldehyde (Aldrich) and 4% sucrose in PBS for 20 minutes, and dehydrated with increasing concentrations of ethanol (30% - 100%) for a total time of 2 h, followed by 5 minutes with hexamethyl-disilazane (Sigma). After drying, samples were covered with a gold layer of a few angstroms thick and imaged with an acceleration voltage of 1 kV.
6.2.11 Atomic Force Microscopy (AFM)

AFM images were acquired using an Asylum Research MFP-3D Atomic Force Microscope for measuring surface roughness. Standard silicon cantilevers, AC240TS (Olympus Optical Co. Ltd.), were used in alternating current mode at a frequency of 72 kHz and a scan rate of 0.75 Hz. Root mean square (RMS) surface roughness was measured using MFP-3D software written in Igor Pro 5 (WaveMetrics Inc.).

6.3 Results and Discussion

6.3.1 NGF Immobilization

Polypyrrole (PPy) has been proposed as an attractive material for neuron interfacing because of its electrical properties. Neurons not only respond to an electrical stimulus, but to a great variety of other factors, including chemical signals mediated by ligand-receptor complexes. As a consequence, the ability to modify biomaterials, including polypyrrole, to present more than one stimulus simultaneously could represent a significant advantage over simpler materials. Following this idea, we report our efforts to immobilize NGF on PPy. The conjugation approach we have applied consists of using phenyl-azido-containing compounds. This technique has been used in numerous studies to nonspecifically create covalent bonds between a compound containing the azido functional group and a substrate, including NGF [36,37,41,43,46-49]. In this type of photochemistry, aryl azides create highly reactive singlet or triplet nitrenes after irradiation, which can subsequently insert into a variety of bonds, including carbon-hydrogen and nitrogen-hydrogen bonds [44,45]. Taking advantage of this high nonspecific reactivity, we successfully used this approach to immobilize NGF on PPy.
The NGF immobilization process is depicted in Figure 6.1. An initial conjugation step included the acylation of the azido-photolinker with PAA, an amine-containing polymer capable of reacting with the succinimide groups of the photolinker and also known to enhance cell adhesion. The product of this conjugation was a photosensitive polymer (PAA-azido) containing phenyl-azido functional groups, confirmed by an absorbance peak at 280 nm. By casting an aqueous solution of PAA-azido and a superimposed layer of NGF, the protein could be immobilized by UV light activating the azido groups to create covalent bonds with both the protein and the substrate. Because of the multiple insertion mechanisms that singlet nitrenes can undergo, the covalent bonds with PPy and NGF could form in a variety of ways, such as via C-H or N-H insertion.

Figure 6.1. Scheme of the NGF immobilization process on PPy. PAA was conjugated to an azido compound (PAA-azido). This conjugate was cast twice on PPy, followed by casting of NGF. UV light exposure promoted the formation of covalent bonds via the azido groups, immobilizing NGF to PPy.

The efficiency of this reaction was analyzed using fluorescence microscopy. NGF was conjugated to FITC to render a fluorescent protein (NGF-FITC), which was only used for detection purposes and not for cell culture
because of reduced bioactivity. Using this fluorescent conjugate it was possible not only to determine the presence of the growth factor after the immobilization process, but also to estimate the surface concentration. For this semi-quantitative analysis, known concentrations of NGF-FITC were cast on PPy, fluorescence images were captured and their intensity determined. A calibration curve relating fluorescence intensity to surface concentration (ng/mm$^2$) (Figure 6.2D) was established and subsequently used to analyze the experimental samples. This method only provided an overall estimation of the surface concentration due to the non-uniform distribution of the protein on both the standards and the samples.

Figure 6.2 illustrates fluorescence images of NGF-FITC immobilized via PAA-azido on PPy. By changing the concentration of protein cast, it was also possible to modulate the concentration of immobilized protein. In particular, 0.5 - 2 µg total protein added to the reaction yielded approximate surface concentrations between 0.2 and 1 ng/mm$^2$ (determined from average of fluorescence intensity in Figure 6.2E and compared to calibration curve). These values are similar to previous reports where 6 fg/µm$^2$ (6 ng/mm$^2$) of NGF were immobilized on beads that successfully produced turning of DRG neurons [65]. The non-linearity of the results could be a result of the non-uniform distribution of the protein and the analysis itself, as the calibration curve is non-linear also (Figure 6.2D). Although control samples with no UV light exposure revealed a relatively high nonspecific binding (Figure 6.2B), the average concentration of nonspecifically adsorbed NGF (i.e., fluorescence intensity) was still statistically lower than the concentration on UV-exposed samples ($P = 1 \times 10^{-4}$) (Figure 6.2E), which confirmed the effective activation of the azido groups. However, the reaction efficiency was low. At most, for an area of 1 cm$^2$, the amount of immobilized protein corresponded to 100 ng, which was approximately 5% of the cast protein. Similar values are reported for this chemistry in the literature [33].
6.3.2 PPy-NGF Characterization

Conductivity measurements were performed to analyze the changes in the electrical properties of the polymer as a result of the immobilization procedure. PPy-NGF had a conductivity of 9.3 ± 2 S-cm\(^{-1}\) which represented a decrease of 30% compared to the value of 14.5 ± 2 S-cm\(^{-1}\) for PPy only (not statistically different, P = 0.12 using t-test). PPy-NGF retained the electrical conductivity and the reduction was comparable with values from other surface chemistries, which vary between 16% and 60% depending on the amount of functionalization [16,23,25]. Furthermore, this decrease in conductivity is minimal compared to differences in orders of magnitude found for PPy doped uniquely with biomolecules (~10\(^{-3}\) S-cm\(^{-1}\)) [20] or for PPy derivatives synthesized with modified monomers having functional groups (~10\(^{-6}\) S-cm\(^{-1}\)) [52].

Stability studies were performed in PBS at 37°C to analyze the amount of NGF leaching out from the surface over time, as a result of nonspecific binding of the protein. This was an important factor to consider to ensure that the observed effects on cells were caused by the immobilized and not the soluble form of NGF. Using a standard sandwich ELISA assay, we analyzed sample volumes of PPy-NGF substrates, including slides with Plexiglas chambers for electrical stimulation, incubated in PBS at 37°C for 72 h. The average concentration of NGF leached into solution was 1.06 ± 0.28 ng/mL and 1.02 ± 0.21 ng/mL (n = 3), for PPy-NGF on tissue culture dishes and inside Plexiglas chambers respectively; this concentration of NGF has been previously shown to have negligible cellular effects [53].
Figure 6.2. Fluorescence images of PPy-NGF-FITC. A) Polypyrrole only; B) Control sample without UV exposure and washes (1 µg of NGF-FITC added); C) Experimental sample with UV exposure and washes (1 µg of NGF-FITC added); D) Calibration curve for different concentrations of NGF-FITC on PPy (error bars correspond to standard deviation); E) Quantitative analysis of fluorescence intensity for samples with different amounts of NGF-FITC added and controls (average ± SEM). Nonspecific binding control was performed without UV exposure for 1 µg of NGF-FITC added. The values in parenthesis correspond to the surface concentrations determined by comparing the average of the fluorescence intensity with calibration curve in D. *, **, ***Statistically significant difference with respect to PPy, 0.5 and 1 µg, respectively, using t-test with P < 0.0005. # Statistically significant difference with respect to nonspecific binding control with P < 0.05. Scale bar = 200 µm.
To further confirm the presence of the immobilized protein, XPS analysis was performed on treated and untreated PPy substrates (Figure 6.3). High resolution spectra of C1s (Figures 6.3A and 6.3C) revealed a main peak for C-C polymer backbone at 285.2 eV and a peak at 286.2 eV attributed to C-N species. After the immobilization process, the C-N contribution increased and a new peak at 288.3 eV appeared, which corresponds to N-C=O species. The increase in C-N was expected from the presence of both the protein and PAA, whereas the peak at 288.3 eV was believed to correspond to amide bonds from NGF. The deconvoluted N1s core level spectra showed two peaks for PPy corresponding to N-H species (400.2 eV), and one attributed to positively charged nitrogen C-N⁺ (401.7 eV) [23,54-56]. The modified polymer with NGF had three main peaks, one for N-H, one for C-N⁺ and one attributed to amide bonds (399.5 eV) [57]. The increase in the C-N⁺ contribution is not expected to be produced by the protein itself, as low levels of quaternary amines have been reported for proteins [58], and this peak was absent in spectra for positive control samples with cast protein (without washing). This suggests that the significant increase in C-N⁺ species could correspond to a modification of PPy with NGF tethering. We speculate that this increase might be a product of coupling of PAA via the nitrogen in the pyrrole ring and the corresponding formation of quaternary amines. An XPS semi-quantitative analysis for finding N1s/C1s ratios also corroborated the presence of PAA and NGF, which revealed an increasing trend in the N1s/C1s ratio with 0.03 for PPy, 0.08 for PPy with PAA only, and 0.10 - 0.13 for PPy-NGF with 1 - 2 µg total protein cast.
Figure 6.3. XPS spectra of PPy (A,C) and PPy-NGF (B,D). High resolution spectra of A) C1s of PPy only; B) C1s of PPy-NGF; C) N1s of PPy only; D) N1s of PPy-NGF. Both carbon and nitrogen spectra showed the increase in C-N and N-C=O peaks, as a result of the immobilized protein.

The immobilization process also produced a noticeable change in the surface topography of PPy as illustrated in Figure 6.4. Wrinkling of the surface was apparent at a macroscopic level, probably because of stresses induced by the drying steps and the conjugation chemistry [59]. In addition, there was also a slight increase in roughness at the microscopic level due to the presence of PPA-Azido and NGF, as evidenced by the presence of bigger and more globular nodules on the surface in both SEM and AFM images. AFM measurements supported this increase, giving an RMS roughness of 60.0 nm and 14.6 nm for PPy-NGF and PPy, respectively. Roughness plays a role in cell interfacing, as it has been shown to affect cell adhesion [60-62]. An increase in surface area has also been reported to be beneficial for neural electrodes [9]. As a result, PC12 cells spread more on PPy-NGF than on PPy (Figure 6.5), probably the result of a combination of the positively charged PAA layer and increased roughness. This
represents a more intimate contact between cells and the polymer, which could be beneficial for either recording or stimulating neurons [3].

**Figure 6.4.** SEM and AFM of PPy and PPy-NGF. A) and B) SEM images of PPy only (Magnification = 1 KX and 5 KX, respectively); D) and E) SEM images of PPy-NGF (Magnification = 1 KX and 5 KX, respectively); C) and F) AFM images of a 5 µm x 5 µm scan for PPy and PPy-NGF, respectively (images obtained by John Slater from Wolfgang Frey group). The increase in surface roughness is a consequence of the immobilization procedure. Scale bars = 10 µm (A,D), 1.5 µm (B,E).
Figure 6.5. Phase-contrast and SEM images of PC12 cells on PPy and PPy-NGF. A) and B) PC12 cells on PPy; C) and D) PC12 cells on PPy-NGF. Cells spread more on PPy-NGF as a result of increased roughness and the presence of PAA. Scale bars = 20 µm (A,C), 10 µm (B,D).

6.3.3 PC12 Cell and DRG Neuron Neurite Extension

PC12 cells are neuron-like cells that differentiate and extend processes in the presence of NGF, and have been extensively studied for the effects and signaling of NGF [63,64]. Figures 6.6 and 6.7 illustrate neurite extension results for these cells cultured on PPy-NGF with different NGF surface concentrations, after 2 or 10 days in culture, respectively (see all conditions in Table 6.1). As controls, cells cultured on PPy with PAA-azido only (no immobilized NGF) were studied with and without NGF in the medium, in order to guarantee similar roughness and surface charge in all samples and rule out any possible advantages from these. Median neurite length and the percentage of cells with neurites were calculated and summarized in Table 6.1.
Figure 6.6. Neurite extension from PC12 cells on PPy-NGF after 2 days in culture. A) Percentage of cells with neurites longer than one cell body (average ± SEM). PAA-azido corresponds to PPy modified with only PAA-azido but no NGF. NGF in solution corresponds to PPy-PAA-azido with soluble NGF. No neurites were observed for PPy-PAA-azido samples; B) Neurite length histogram for PC12 cells cultured on PPy-NGF with 0.98 ng/mm² (2 μg of NGF added); C) Neurite length histogram for PC12 cells cultured on PPy-PAA-azido with soluble NGF (50 ng/mL). D)-F) Representative fluorescence images of PC12 cells cultured on PPy-PAA-azido, PPy-NGF (0.98 ng/mm²) and PPy-PAA-azido with soluble NGF (50 ng/mL), respectively. Cells were primed with NGF in solution for one week prior to experiments, seeded on substrates and fixed and stained with phalloidin-TRITC after 2 days in culture. Scale bar = 50 μm.
Figure 6.7. Neurite extension from PC12 cells on PPy-NGF after 10 days in culture. A) Percentage of cells with neurites longer than one cell body (average ± SEM). PAA-azido corresponds to PPy modified with only PAA-azido but no NGF. NGF in solution corresponds to PPy-PAA-azido with soluble NGF; B) Neurite length histogram for PC12 cells cultured on PPy-NGF with 0.98 ng/mm² (2 µg of NGF added); C) Neurite length histogram for PC12 cells cultured on PPy-PAA-azido with soluble NGF (50 ng/mL).

As suggested by the results, PC12 cells could successfully grow neurites on PPy with immobilized NGF. After 2 days in culture, PC12 cells cultured on PPy-NGF (2 µg NGF added to reaction, 0.98 ng/mm²) extended neurites with similar lengths (median length = 16.8 µm) as controls with PAA-azido and soluble
NGF (median length = 17.2 µm). Furthermore, when the percentage of cells with neurites was analyzed, a value of ~ 6% was obtained for all concentrations of immobilized NGF, as well as for soluble NGF (50 ng/mL) (Figure 6.6A). It is important to emphasize that these results were not a product of soluble NGF leaching out of the surface, as controls with PAA-azido and 1 ng/mL of soluble NGF (same concentration as detected with ELISA from stability studies) did not exhibit any significant neurite extension (Table 6.1).

Table 6.1. PC12 Cell Neurite Extension on PPy-NGF

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Median Length (µm) (n) a</th>
<th>Percentage of Cells with Neurites (n ) b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No Electrical Stimulation</strong> c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Days in Culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPy-PAA-azido</td>
<td>N/A</td>
<td>0 (570)</td>
</tr>
<tr>
<td>PPy-NGF (0.5 µg)</td>
<td>13.5 (636)</td>
<td>5.52 ± 0.49 (945)</td>
</tr>
<tr>
<td>PPy-NGF (1 µg)</td>
<td>13.4 (770)</td>
<td>6.18 ± 1.50 (1333)</td>
</tr>
<tr>
<td>PPy-NGF (2 µg)</td>
<td>16.8 (1030)</td>
<td>6.35 ± 1.74 (1730)</td>
</tr>
<tr>
<td>PPy-PAA-azido + sol. NGF (1 ng/mL)</td>
<td>9.2 (622)</td>
<td>0.51 ± 0.18 (1229)</td>
</tr>
<tr>
<td>PPy-PAA-azido + sol. NGF (50 ng/mL)</td>
<td>17.2 (651)</td>
<td>6.55 ± 0.27 (932)</td>
</tr>
<tr>
<td>10 Days in Culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPy-PAA-azido</td>
<td>N/A</td>
<td>0.52 ± 0.16 (715)</td>
</tr>
<tr>
<td>PPy-NGF (0.5 µg)</td>
<td>8.4 (207)</td>
<td>2.06 ± 1.00 (859)</td>
</tr>
<tr>
<td>PPy-NGF (1 µg)</td>
<td>11.6 (451)</td>
<td>4.38 ± 2.40 (1979)</td>
</tr>
<tr>
<td>PPy-NGF (2 µg)</td>
<td>42.2 (556)</td>
<td>8.95 ± 1.45 (3825)</td>
</tr>
<tr>
<td>PPy-PAA-azido + sol. NGF (50 ng/mL)</td>
<td>42.1 (246)</td>
<td>13.74 ± 0.58 (1056)</td>
</tr>
<tr>
<td><strong>Electrical Stimulation</strong> d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPy-NGF (2 µg) with electrical stimulation</td>
<td>12.0 (1383)</td>
<td>1.44 ± 0.24 (3798)</td>
</tr>
<tr>
<td>PPy-NGF (2 µg) without electrical stimulation</td>
<td>8.0 (765)</td>
<td>0.30 ± 0.15 (2963)</td>
</tr>
</tbody>
</table>

a Neurite distribution was obtained (non-Gaussian) and the median length is reported. n = total number of neurites analyzed
b Cells with neurites longer than one cell body (average ± SEM of three samples). n = total number of cells analyzed
c Cells were primed with soluble NGF for 1 week
d Cells were primed with soluble NGF for 24 h and analyzed after 2 days as previously described in [10] N/A No neurite histogram was obtained because very few neurites were observed
In addition, we also confirmed that PPy-NGF was not promoting additional adsorption of serum proteins from the medium that could indirectly enhance neurite extension. We repeated experiments for PPy-NGF with 0.98 ng/mm$^2$ in serum-free conditions, and found that the median length was 18.02 µm ($n = 776$). This actually represents a small but not statistically significant increase with respect to serum-containing media experiments, which suggests that protein adsorption on the surface only masked the immobilized NGF to a small extent.

PC12 cell neurite extension was also analyzed at a longer time point, 10 days, to further investigate the enduring effect of immobilized NGF. As summarized in Table 6.1 and Figure 6.7, immobilized NGF still supported neurite extension for a surface concentration of 0.98 ng/mm$^2$ (median length = 42.2 µm), but was significantly decreased for 0.28 ng/mm$^2$ (median length = 11.6 µm) and 0.21 ng/mm$^2$ (median length = 8.4 µm). More importantly, the percentage of cells having neurites at this time-point was statistically lower for all NGF surface concentrations ($P = 0.004, 0.03, 0.05$ for 0.21, 0.28, 0.98 ng/mm$^2$, respectively), when compared to controls on PAA-azido with soluble NGF (Figure 6.7A). These results suggest a possible detrimental effect resulting from the inhibition of NGF endocytosis and signaling (e.g., for differentiation [31]), which at the same time might not dramatically interfere with enhanced localized actin polymerization at the growth cone.

NGF has been immobilized with retained activity in many other studies, and effects on PC12 cells have been already reported by other groups [33,34,36-40]. Here, we observed similar results for primed PC12 cells cultured on PPy-NGF, which exhibited comparable neurite extension to controls with soluble NGF for short-time points (2 days). However, we also observed some important differences for longer time points (10 days). Although neurite elongation was very similar between the highest concentration of immobilized NGF and the soluble
form, there was a significant decrease in the total number of cells actually having neurites. We hypothesize that this discrepancy is a result of inhibition of endosome signaling by tethered NGF.

Previous studies have shown that after NGF binds to TrkA receptors on the cellular membrane, the receptor-ligand complex is internalized and transported to the cell body, which triggers intracellular signaling cascades [64]. However, not all signaling is dependent on internalization. Zhang et al. reported dramatic differences in intracellular pathways for internalized receptors versus "surface receptors" [31]. This group demonstrated that non-internalized receptors exhibited phosphorylation of tyrosines after NGF binding that triggered a long-lasting activation of the PI 3- kinase pathway that is critical for survival. In contrast, they also demonstrated that endocytosis was essential for differentiation, and therefore neurite extension in PC12 cells, as these cells only extend neurites when differentiated. However, it has also been shown that PI 3-kinase regulates neurite extension. Gallo et al. showed that axon sprouting on NGF-coated beads was dependent on PI 3-kinase activity [34], and more recently, Aoki et al. demonstrated that activation of Cdc42 and Rac1, GTPases that modulate actin polymerization, was locally controlled by PI 3-kinase [35].

In light of these studies, we hypothesize that in early time points, primed PC12 cells are capable of easily extending neurites as a result of the priming (i.e., cells are already differentiated with soluble NGF for a week) and the local activation of PI-3 kinase pathway by immobilized NGF. However, for longer time points, two opposite effects could be present: 1) decreased differentiation and neurite extension as a result of endosome inhibition, and 2) enhanced activation of PI-3 kinase pathways, which would locally stimulate actin reorganization for neurite growth. The combination of these two factors could explain the decrease
in cells having neurites after 10 days in culture on PPy-NGF, but the similar neurite lengths of these processes.

As PC12 cells are not able to extend neurites without being differentiated, and this appears to depend on receptor endocytosis, we reconfirmed the proposed localized activation of neurite growth by PPy-NGF with another cell type that could extend neurites without having conflicting effects on the endocytosis pathway. Rat DRG neurons were cultured on PPy-NGF and controls and analyzed again for neurite extension after 2 days in culture. As depicted in Figure 6.8, PPy-NGF also promoted neurite outgrowth in DRG neurons when compared to PPy-PAA-azido only, and the response was only slightly smaller and not statistically different than soluble NGF controls. This further supported the fact that immobilized NGF was active and capable of increasing neurite outgrowth from neurons.

6.3.4 PC12 Cell Neurite Extension with Electrical Stimulation

Previous studies on PPy have shown an increase in neurite length with electrical stimulation [10]. Because of these results, we wanted to explore if this effect was also observed with PPy-NGF. For these experiments, electrical stimulation protocols were exactly followed as previously published [10]. As shown in Table 6.1, there was an enhancement in neurite length for PPy-NGF with electrical stimulation compared to PPy-NGF without stimulation (12 µm and 8 µm, respectively; statistically different for P<0.001). This result suggests an additive effect of electrical stimulation and chemical stimulation provided by the immobilized NGF. However, the median neurite length on PPy-NGF was lower than for unmodified PPy controls with NGF in solution. In that case, electrically-stimulated cells had a median length of 17 µm (n = 317), whereas cells with no stimulation had a median length of 8 µm (n = 244).
A possible explanation for this difference in median length when compared to controls could be again that PC12 cells exhibited restricted responses to immobilized NGF because of endosome signaling inhibition. Also, although the chemical grafting is a surface modification, there is a slight decrease in conductivity that correlates to a decrease in current passed through PPy-NGF,
specially on the surface, compared to the current passed through unmodified PPy. However, it is important to emphasize that although the increase in neurite length with electrical stimulation on PPy-NGF was small, it was still statistically significant, which again suggests a positive impact from the combination of stimuli. Further optimization of electrical stimulation protocols will be necessary to maximize neurite extension.

The effects of electrical stimulation on neurite growth are still unclear. In these studies, current was continuously passed through PPy-NGF slides for 2 h at a constant voltage of 100 mV, as previously described [10]. Although the mechanism for enhanced cell growth in these experiments is not known, this stimulation is different from conventional electrical stimulation with electrodes, where higher voltages or currents are used for very short pulses with the purpose of depolarizing cells or producing action potentials [65]. In our case, electrical stimulation was constant for a longer time, which has been shown to have multiple effects, for example on protein adsorption [13]. Patel and Poo also described in an early paper the effect of steady electric fields in neurite growth, and explained that electric fields of 0.10 - 10 V/cm produced intracellular potential differences of 0.01 - 1 µV and transmembrane potentials of 0.5 – 5 mV, which could produce continuous movement of charged molecules inside and outside the cell and modulate neuron behavior [66]. In this same paper, the authors also suggested the effect of the electric field on surface glycoproteins. More recently, Howe showed that depolarization with high potassium chloride solutions increased the phosphorylation of TrkA receptors and neurite growth [67], which could have some influence in our experiments, although the degree of depolarization in our studies is probably very small.

Immobilization of NGF on PPy represents a significant modification of this biomaterial. Although electrical stimulation is a key factor to include in nerve repair therapies, it is desirable to include multiple cues in order to maximize cell
responses. Growth factors such as NGF are important mediators that trigger a variety of cell behaviors. It is believed that the presence of NGF for nerve regeneration therapies might be crucial, and attempts to deliver it by different methods have been explored [68-70]. Similarly, released NGF has also been used for neural probes to improve interfacing between the cells and the electrodes [3]. Although NGF is a soluble factor, it has been immobilized with retained activity in several studies [33,34,36-40]. The immobilized biomolecule could provide a more stable and long-lasting stimulus, as opposed to the release of soluble NGF, which might be drastically affected by rapid degradation. Even more, it has been proposed that cellular responses to immobilized growth factors are enhanced as a result of inhibition of down-regulation processes [71]. Therefore, the immobilization of NGF could be an alternative method for the presentation of this protein for neural applications. With respect to PPy itself, the use of conducting polymers for nerve regeneration and electrodes has been proposed for several years [4-14]. Here, we combined these two important stimuli to render a conducting polymer bioactive, which enhanced neurite outgrowth in vitro.

6.4 Conclusion

NGF was successfully immobilized on PPy using PAA-azido as a linking compound. Three different surface concentrations were studied, and it was determined that a surface concentration of 0.98 ng/mm² produced similar neurite extension in PC12 cells compared to soluble NGF (50 µg/mL) after 2 days in culture. However, although immobilized NGF maintained the effect on neurite length after 10 days in culture, some detrimental effects were observed as a potential consequence of inhibition of endocytosis-dependent pathways. In addition, electrical stimulation using PPy-NGF further increased neurite length with respect to controls without stimulation. This suggested a possible additive
effect of electrical and chemical stimuli, both exclusively provided by either the
bulk or the surface properties of the material. Cell adhesion was also improved
as a consequence of the positively charged PAA and the increased roughness
from the immobilization procedure. This modified material could potentially be
used for nerve regeneration strategies such as nerve conduits and neural
electrodes to improve chemical interfacing with neurons.

6.5 References

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Chapter 7
In search of Combinatorial Strategy III: Fabrication of PPy Microchannels

Introduction

Chapter 5 and 6 described the studies on two combinatorial strategies. Based on the results from these two investigations, the combination of multiple extracellular cues on modified biomaterials definitively has a beneficial impact on neuronal behavior. Because of this, we hypothesize that a triple combination including electrical, chemical and physical stimulation could have an even stronger effect on cells. To implement this triple stimulation, we propose to create polypyrrole microchannels, modify the surface with immobilized NGF and perform electrical stimulation experiments on cells. This chapter describes the first step toward this ultimate goal, which is the fabrication of microchannels in polypyrrole.

7.1 Background and Motivation

Polypyrrole (PPy), a biocompatible electrically-conductive polymer, is extensively used in biological applications such as neural electrodes [1-3], and scaffolds for nerve regeneration in neural tissue engineering [4-6]. PPy is an attractive material for neural probes and nerve guidance channels because of its biocompatibility, good conductivity, ease of synthesis and the potential to have high surface areas [3,7]. However, it is still desired for these and other biomedical applications to provide chemical and physical guidance cues in conjunction with electroactivity provided by the conducting polymer.

Surface modification of conducting polymers for incorporating biological cues involves different approaches that impact chemical and physical properties
of the polymer. Chemical modification has been extensively investigated using biomolecules as dopants [8-10], or immobilizing bioactive moieties on the surface of the material [2,11-13]. Physical modification has been explored by increasing surface roughness using various methods such as creation of microporous films using polystyrene spheres templates [14], fabrication of composites of nanoparticles and polylactide [15], growing conducting polymers within hydrogels [16], and blending with biomolecules to yield “fuzzy” structures [8].

We reported covalent immobilization of nerve growth factor (NGF) on the surface of PPy in Chapter 6. Combination of this bioactive surface with electrical stimulation provided by PPy was showed to enhance neurite extension. In Chapter 5 we showed that poly(dimethyl siloxane) (PDMS) microchannels with immobilized NGF effectively increased neuronal polarization (i.e., axon formation) and axon length of neurons. Combination of physical cues (i.e., microchannels) and chemical guidance (i.e., immobilized protein) was essential for favorable neuronal responses. Based on these results, we hypothesize that a conducting polymer with both physical and chemical guidance cues could further improve axon formation and growth. Fabrication of PPy microchannels as described here provides both physical and electrical cues to neurons, which can be subsequently combined with immobilized NGF to enhance neuronal behavior.

In an effort to modify PPy with topographical features, we report the fabrication of 1 and 2 µm wide microchannels using electron beam (e-beam) lithography and electropolymerization. Although PPy patterning for microelectronic applications has been extensively reported in the literature [27-37], patterns smaller than 5 µm (relevant size for many cell functions) have been scarcely studied. More importantly, biological applications of small PPy patterns (i.e., less than 5 µm) have not been investigated. Additionally, there are no previous studies on the effect of patterning and polymerization techniques on
pattern size (e.g., width and depth), morphology and surface roughness, important parameters for cellular applications.

E-beam writing and polymerization conditions were found to drastically affect the morphology of the microchannels, determining the depth, roughness and sharpness of the patterns. We investigated a range of electropolymerization conditions including monomer and dopant concentration (12.5 - 100 mM), polymerization current (36 - 720 µA) and polymerization time. For higher monomer concentration, larger current and longer polymerization time, the channels were deeper and rougher, and the geometry was less defined.

We investigated the effect of the fabricated PPy microchannels on polarization and axon length of hippocampal cells, an extensively used model for this type of behavior of neurons [19,20]. Microchannels were shown to have a significant effect on neuron polarization with a two-fold increase in the number of neurons with axons, whereas the effect of topography on axon length is negligible. Microchannels also have an effect on the orientation of axon growth, promoting parallel or perpendicular alignment with respect to the microchannels. These novel results demonstrate that controlled PPy patterning with small dimensions can be applied to cellular and biomedical purposes, in addition to the traditional microelectronic applications.

7.2 Materials and Methods

7.2.1 Microfabrication of PPy Channels

Microchannels 1 and 2 µm wide and 50 -2000 nm deep were created on PPy using microfabrication techniques. The procedure had two steps: 1)
patterned resist using electron beam (e-beam) lithography, and 2) electropolymerization of PPy on the patterns (see Figure 7.1).

Micropatterns were written with e-beam lithography. Indium tin oxide (ITO) (conducting material) coated glass slides (Delta Technologies) were cut into 4 cm²-pieces and spin coated with 1% poly(methyl methacrylate) PMMA resist in anisole (Microchem) for 45 seconds and 3000 rpm, and baked for 1 h at 160°C. Microchannels of 1 or 2 µm were written on the PMMA resist using e-beam (Raith-50 and XL-30 SEM, LaB₆ source) with an area dose of 220 µAs/cm² and beam current of 0.1 nA. After the patterns were written, electron-exposed areas were developed with a mixture of methyl-isobutyl-ketone and isopropyl alcohol (1:3) (Sigma) for 1 minute, followed by 1 minute in isopropyl alcohol, exposing the conducting ITO on those areas. For thick resist experiments, 2% PMMA resist in chlorobenzene was spin-coated with the same conditions.

PPy microchannels were polymerized electrochemically, a common technique to synthesize PPy thin films [4]. Patterned ITO coated slides were used for the galvanostatic deposition of the conducting polymer. Specifically, a three-electrode cell was used, which consisted of the ITO slide as the working electrode, a platinum gauze counter electrode and a saturated calomel reference electrode. The polymer was deposited from an aqueous solution of 12.5 - 100 mM pyrrole monomer (Aldrich) and 12.5 - 100 mM sodium salt of poly(styrene sulfonate) (PSS) (Aldrich), using an offset voltage of 720 mV and currents of 36 - 720 μA (see Table 7.1). A Pine Instruments AFRDE5 bipotentiostat was used as the DC voltage source. Microchannels were polymerized for 10 or 30 seconds as stated in Table 7.1.

7.2.2 Synthesis of PPy Thin Films (Unmodified PPy Controls)

PPy thin films were synthesized as controls without patterns for cell culture. Thin films were electrochemically synthesized on plain ITO coated slides...
with the same three-electrode cell used for PPy microchannels, using an aqueous solution of 25 mM pyrrole monomer and 25 mM PSS, an offset voltage of 720 mV and current of 7.2 mA. Higher polymerization current was required to polymerize thin films, as the area available for polymerization was much larger (~6 cm X 2 cm) than for microchannels. Thin films were subsequently cut in 1 cm² pieces, and used for cell culture.

7.2.3 Microfabrication of PDMS Channels

Microchannels 1 and 2 µm wide and 400 nm deep were created on PDMS using soft lithography techniques as described in Chapter 4. Briefly, e-beam lithography was used to write patterns on 4% PMMA resist in chlorobenzene (Microchem), which was spin coated on silicon wafers with a 4-µm-thick SiO₂ layer. After writing and development, lift-off was performed by thermal evaporation (Denton) of a 100 nm chromium film (R.D. Mathis) and acetone stripping. Reactive Ion Etching (RIE) of the SiO₂ layer was performed in an etcher (Plasma Technology) with a mixture of CHF₃ and oxygen. The etched wafer was silanized with tridecafluoro-1,1,2,2-(tetrahydrooctyl) trichlorosilane (Gelest), and subsequently used as a master for replica molding of the PDMS (Sylgard 184, Dow Corning) substrates. PDMS substrates with microchannels were coated with a layer of photoreactive polyallylamine as previously described [18], which rendered the material cell-adhesive for hippocampal neuron culture.

7.2.4 Hippocampal Cell Culture and Immunochemistry

For cell culture, PPy substrates (microchannels and thin films) were subsequently transferred to sterile 3 cm tissue culture dishes, UV-sterilized, incubated with poly-D-lysine (Sigma) for 2 h, washed twice with sterile water, air dried and stored at 4°C. Embryonic rat hippocampal cells (E18) were isolated
from commercially-obtained hippocampus tissue (BrainBits). The hippocampi were incubated with papain (Worthington) in Hibernate E medium (Brainbits) (4 mg/mL) at 30°C for 20 minutes, followed by physical trituration with a fire-polished Pasteur pipette. Cells were counted and plated on PPy substrates at 7.5 x 10³ cells/cm², and cultured with Neurobasal Medium (Invitrogen) supplemented with B-27 (Invitrogen), L-glutamine (Fisher, 0.5 mM), L-glutamic acid (Sigma, 25 µM) and 1% antibiotic-antimycotic (Sigma, 10,000 units/mL of penicillin, 10 mg/mL of streptomycin and 25 µg/mL of amphotericin).

Cells were incubated on substrates with PPy microchannels and PPy thin films as controls. After 20 h in culture at 37°C and 5% CO₂, cells were fixed with 4% paraformaldehyde (Sigma), 4% sucrose (Fisher) in PBS for 20 minutes, followed by permeabilization for 20 minutes with 0.1% Triton-X100 (Sigma) in 2% bovine serum albumin (BSA) (Jackson ImmunoResearch) in PBS (PBS-BSA), and blocking for 1 h at 37°C with 2% PBS-BSA. Samples were incubated with an antibody for Tau-1 (axonal marker) (Chemicon, 1:200) in 2% PBS-BSA overnight at 4°C, followed by incubation with a fluorescently-labeled secondary antibody (Alexa 488-conjugated goat anti-mouse, Molecular Probes), for 1 h at 37°C. Fluorescence images were captured with an inverted phase-contrast and fluorescence microscope (IX-70, Olympus), using a color CCD video camera (Optronics MagnaFire, model S60800) and analyzed using Image J (NIH). All cell experiments were repeated five times on different days. Experiments on PDMS substrates were performed following the same protocol.

7.2.5 Polarization and Axon Length Analysis

Based on published criteria, a hippocampal cell was defined as polarized (stage 3) when one of its neurites was at least twice as long as the other neurites and it stained positively for Tau-1 [20-22]. The fraction of polarized cells was defined as the ratio of cells with axons to the total number of cells per sample as
analyzed from fluorescence images. In the same experiments, axon length and angle were also measured and defined as the straight-line distance from the tip of the axon to the junction between the cell body and axon base, and the angle between this line and a 0° line in the direction of the microchannels (see inset in Figure 7.5C). In the case of branched axons, the length of the longest branch was measured from the tip of the axon to the cell body, and then each branch was measured from the tip of the axon to the branch point. An average of 130 cells were analyzed per sample for each condition, and the average and standard error of the mean (SEM) were calculated for all data. P-values for fraction of polarized cells and axon length data were analyzed using 2-sided Student’s t-test with respect to controls on PPy films. Statistical significance was determined for P < 0.05.

7.2.6 Scanning Electron Microscopy (SEM)

Microchannel characteristics and axon extension were analyzed with a LEO 1530 scanning electron microscope. Microchannels were imaged with a typical acceleration voltage of 10 kV. Microchannel depth was determined from SEM images by analyzing cross sections of the patterns (n = 3). For imaging neurons, cells were fixed with 4% paraformaldehyde (Aldrich) and 4% sucrose in PBS for 20 minutes, and dehydrated with increasing concentrations of ethanol (30% - 100%) for a total time of 2 h, followed by 5 minutes exposure to hexamethyl-disilazane (Sigma). After drying, samples were sputter coated with a gold layer and imaged with an acceleration voltage of 1 kV.

7.2.7 Atomic Force Microscopy (AFM)

AFM images were acquired using an Asylum Research MFP-3D Atomic Force Microscope for measuring PPy surface roughness. Standard silicon nitride
cantilevers, model OTR8 (Veeco Nanoprobe), were used in contact mode at a scan rate of 0.5 Hz. 512 x 512 scans of 2 X 2 μm sections on top of the PPy channels were used for calculating root mean square (RMS) surface roughness using MFP-3D software written in Igor Pro 5 (WaveMetrics Inc.). Larger overview scans (50 x 50 μm) were also acquired with 512 x 512 resolution with 0.5 Hz scan rate.

7.3 Results and Discussion

7.3.1 Fabrication and Characterization of PPy Microchannels

PPy microchannels were fabricated taking advantage of the electropolymerization process. In this polymerization, pyrrole monomer only polymerizes on substrates that are electrically conductive. This technique is widely used for performing polymer synthesis using a metal or ITO-coated substrate [23]. Based on this principle, the fabrication of PPy microchannels was performed by first patterning an insulating resist on a conductive substrate using e-beam lithography, a direct-writing technique [24], and then polymerizing on the exposed conductive areas (Figure 7.1). Other techniques such as patterning of self-assembled monolayers also create insulating patterns on conductive substrates for patterning PPy [30,31].

Growth and morphology of PPy microchannels was strongly dependent on electropolymerization conditions, in particular monomer and dopant concentrations, polymerization current and time. All studied conditions are summarized in Table 7.1, which also includes the depth of the microchannels obtained from SEM images of cross sections, and the roughness obtained from AFM. As illustrated in Figure 7.2, Figure 7.3 and Figure 7.4, polymerization conditions determined the depth, roughness and geometry of the structures. In
general, for larger polymerization currents and higher monomer / dopant concentrations such as in Figures 7.2A – 7.2C, microchannels polymerized rapidly producing deeper and rougher structures. These conditions also produced less-defined geometries, decreasing the gap distance in between subsequent microchannels. For lower polymerization currents and pyrrole /dopant concentrations, the reaction was more controlled, producing smoother PPy structures and maintaining the original dimensions of the ITO patterns. Figure 7.2 illustrates the growth trend for some of the studied conditions, which suggests a larger impact of the polymerization current on microchannel depth. Figure 7.4 shows AFM images of microchannels for two conditions and of thin film controls, which illustrates the increase in roughness with increasing depth. This is the same trend previously reported for PPy thin films [3].

PPy growth is controlled by electric field properties (current or voltage) and solution concentration of monomer and electrolyte [25,44]. Low monomer concentration is known to result in diffusion-limited aggregation growth (i.e., nucleation-limited polymerization), whereas low voltages or currents result in diffusion-limited polymerization growth (i.e., growth rate-limited polymerization) [25]. Correspondingly, nucleation has been classified as instantaneous or progressive, and the growth as two or three dimensional [45]. Low monomer concentration produces fewer nuclei. When the current is also low, the nuclei grow slowly in two dimensions. On the contrary, high monomer concentration induces high nucleation, which is translated in the formation of many PPy particles on the substrate surface. High polymerization currents produce rapid growth of the nuclei in a three dimensional fashion. Combined low monomer concentration and low current produces few PPy nuclei that grow slowly in two dimensions (i.e., thin films, smooth surfaces). High monomer concentration and large current produces many nuclei that grow very rapidly in a three dimensional fashion (i.e., thick films, rough surfaces). These growth characteristics explain
the obtained microchannel morphologies depending on the polymerization conditions.

**Figure 7.1.** Scheme for fabrication of PPy microchannels. An ITO glass slide is spin-coated with a PMMA resist (1). Patterns are written using e-beam lithography (2), developed (3), and electropolymerized in a three-electrode cell with a pyrrole / PSS solution (4). PPy microchannels are created as PPy exclusively polymerizes on the developed conducting areas (5).
Table 7.1. Electropolymerization Conditions and Microchannel Depth and Roughness

<table>
<thead>
<tr>
<th>Pyrrole/PSS Concentration (mM)</th>
<th>Polymerization Current (µA)</th>
<th>Polymerization Time (seconds)</th>
<th>2 µm Microchannel Depth (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1 µm Microchannel Depth (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RMS Roughness (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>144</td>
<td>30</td>
<td>54.1 ± 12.9</td>
<td>51.0 ± 10.5</td>
<td>10.79 ± 1.04</td>
</tr>
<tr>
<td>25</td>
<td>36</td>
<td>30</td>
<td>68.8 ± 11.4</td>
<td>78.8 ± 3.7</td>
<td>11.74 ± 0.26</td>
</tr>
<tr>
<td>25</td>
<td>144</td>
<td>10</td>
<td>275.2 ± 14.8</td>
<td>241.9 ± 36.0</td>
<td>13.66 ± 0.18</td>
</tr>
<tr>
<td>25</td>
<td>144</td>
<td>30</td>
<td>329.4 ± 53.4</td>
<td>296.5 ± 68.1</td>
<td>14.27 ± 0.38</td>
</tr>
<tr>
<td>50</td>
<td>144</td>
<td>30</td>
<td>454.6 ± 89.5</td>
<td>338.7 ± 49.0</td>
<td>15.06 ± 0.29</td>
</tr>
<tr>
<td>50</td>
<td>720</td>
<td>30</td>
<td>1184.1 ± 161.2</td>
<td>1012.3 ± 214.5</td>
<td>20.45 ± 1.78</td>
</tr>
<tr>
<td>100</td>
<td>720</td>
<td>30</td>
<td>2134.4 ± 500.0</td>
<td>1628.8 ± 378.5</td>
<td>33.55 ± 0.71</td>
</tr>
</tbody>
</table>

PPy Thin Films (Controls)

| 25                              | 7200<sup>c</sup>           | 120                          | N/A                                    | N/A                                    | 19.25 ± 2.28                   |

<sup>a</sup> Obtained from cross-section SEM images for n = 3 (average ± SEM); Data analyzed by Jae Y. Lee from Schmidt lab.

<sup>b</sup> Obtained from AFM for n = 3 (average ± SEM); Data analyzed by Jon D. Nickels from Schmidt lab.

<sup>c</sup> Higher current and time were required because of larger polymerization area.
Figure 7.2. PPy microchannel morphology for different electropolymerization conditions (SEM and light microscopy images). Microchannels polymerized with A)-C) 50 mM pyrrole / PSS concentration, 720 µA polymerization current, and 30 seconds of polymerization time (thickness ~900 nm); D) - F) 25 mM pyrrole / PSS concentration, 144 µA polymerization current, and 30 seconds of polymerization time (thickness ~300 nm); G) - I) 12.5 mM pyrrole / PSS concentration, 144 µA polymerization current, and 30 seconds of polymerization time (thickness ~50 nm). All patterns were written with 0.1 nA during e-beam lithography. Polymerization conditions control microchannel depth, roughness and morphology. Scale bar = 5 µm (A,D,G), 2 µm (B,E,H), 10 µm (C,F,I).
Figure 7.3. Effect of pyrrole/PSS concentration and electropolymerization current on PPy microchannel depth. A) Depth of microchannels polymerized with 144 µA, 30 seconds of polymerization time and increasing monomer/dopant concentration; B) Depth of microchannels polymerized with either 25 or 50 mM pyrrole/PSS concentration, 30 seconds of polymerization time and increasing current. Error bars represent SEM.
Figure 7.4. Representative AFM images of PPy microchannels. A) 20 X 20 \( \mu \)m scan of PPy microchannels synthesized with 12.5 mM pyrrole / PSS, 144 \( \mu \)A polymerization current and 30 seconds of polymerization time; B) 2 X 2 \( \mu \)m scan on PPy microchannel ridge with same conditions as A (RMS = 10.79 ± 1.04 nm); C) 20 X 20 \( \mu \)m scan of PPy microchannels synthesized with 50 mM pyrrole / PSS, 720 \( \mu \)A polymerization current and 30 seconds of polymerization time; D) 2 X 2 \( \mu \)m scan of PPy microchannels with same conditions as C (RMS = 20.45 ± 1.78 nm); E) 20 X 20 \( \mu \)m scan of PPy thin film synthesized with 25 mM pyrrole / PSS, 7200 \( \mu \)A polymerization current and 60 seconds of polymerization time; F) 2 X 2 \( \mu \)m scan of PPy microchannels with same conditions as E (RMS = 19.25 ± 2.28 nm). Higher monomer/dopant concentration and larger polymerization current increased PPy microchannel roughness.
E-beam writing current also affected PPy microchannel growth. Figure 7.5 shows patterns written with low currents, which exhibited slow growth. This difference could be a result of the subsequent PMMA resist development, which removes more or less material on the patterns depending on the exposure to the e-beam [26]. Lower current during the writing correlates with less development of the resist, which further translates to more PMMA-protected ITO areas, and therefore less polymerization.

Figure 7.5. SEM photomicrographs of microchannels written with different e-beam currents. A) Microchannels written with a decreasing current ramping from 0.16 nA (left) to 0.02 nA (right), and polymerized with 25 mM pyrrole / PSS concentration, 144 µA polymerization current, and 30 seconds of polymerization time; B) Higher-magnification image of microchannels with different PPy thickness. Higher e-beam currents produced deeper and more defined patterns. Scale bar = 5 µm (A), 2 µm (B).
PPy micropatterning has been extensively explored for microelectronic applications, but not for biological applications. Microcontact printing techniques have been utilized in several applications for the patterning of silanes [27-30] and alkanethiols [31,32]. Patterned silanes can create positive or negative PPy patterns depending on the underlying substrate (pattern size ~15-50 µm) [27,30]. Microcontact printing of aminosilanes has been used to pattern copper first, followed by PPy electrodeposition (pattern size ~2-80 µm) [29]. Alkanethiols can be used for masking on a conductive substrate [31] (pattern size ~10 µm), or pyrrole-thiol compounds can be used to promote selective polymerization (pattern size ~2-10 µm) [32]. Techniques such as conventional photolithography (pattern size ~30 µm) [33], domain separation of block copolymers (pattern size ~50 nm) [34], masking by polymer brushes (pattern size ~50 µm) [35], mold insertion (pattern size ~50 - 300 µm) [36] and photopolymerization (pattern size ~25 µm) [37] have also been explored for patterning PPy.

Many of these PPy patterning techniques have only created patterns with dimensions between 10 -100 µm, and more importantly, none of these studies was performed for biological applications. E-beam lithography allowed patterning of structures on a scale relevant to cell function. In addition, pattern depth and roughness is also important in the biomaterial field, which was not reported in many of these previous studies. We found that although e-beam writing is time-consuming, it allowed fabricating smaller patterns, in addition to better control over polymer growth by modulating writing current and subsequent development of the resist.

Investigations on PPy micropatterning for influencing cell function are very limited. Song et al. [38] reported the fabrication of PPy patterns (pattern size ~100 µm) to show the selective modification of PPy with adhesive molecules that influence cell adhesion. This larger scale patterning could be used to control cell
organization and positioning, whereas the techniques and dimensions described here are used to control axonal outgrowth more directly, which is fundamental for neural engineering applications. In particular, patterning of conducting polymers such as PPy could be used in neural probes or nerve conduits to create surfaces that modulate neuron responses to the implanted device.

Figure 7.6. SEM photomicrographs of microchannels written on different resist thicknesses. A) Microchannels polymerized on a PMMA resist ~ 10 nm thick with 50 mM pyrrole/PSS concentration, 144 µA and 30 seconds (microchannel depth = 338 ± 49 nm); B) Microchannels polymerized on a PMMA resist ~ 100 nm thick with 50 mM pyrrole/PSS concentration, 144 µA and 30 seconds (microchannel depth = 799.3 ± 113 nm). Thicker resist produces deeper and more defined microchannels. Scale bar = 1 µm.
7.3.2 Hippocampal Neuron Response to PPy Microchannels

Embryonic hippocampal neurons were cultured on microstructured and unmodified PPy to determine the effects of topography on polarization and axon elongation mechanisms. One set of PPy patterning conditions was chosen for fabricating well-defined patterns for cell culture. These conditions were e-beam current of 0.1 nA, and electropolymerization with 25 mM pyrrole and PSS, 144 µA and 30 seconds of polymerization.

We reported that 1 - 2 µm wide and 400 - 800 nm deep PDMS microchannels significantly increased the number of stage 3 cells (i.e., polarized neurons) during the first 20 h in culture (Chapter 5). Here, we corroborated and confirmed these results by fabricating similar topographical features on a conducting polymer. As observed in Figure 7.7, hippocampal neurons cultured on PPy microchannels polarized faster, meaning that more cells had defined axons when compared to controls on unmodified PPy. Figure 7.8A illustrates the quantitative analysis of these responses. Both 1 and 2 µm microchannels had a statistically significant increase in the number of polarized cells with respect to cells on unpatterned PPy (P = 0.0007 for 2 µm microchannels; P = 0.009 for 1 µm microchannels). Similar results on PDMS substrates are also shown in this graph, which further confirms the particular effect of the topography itself and not the specific material. It is important to mention that the surface roughness (RMS) of the thin film was ~19 nm, which is larger than the roughness of the microchannels (from Table 7.1, RMS ~14 nm). These data eliminate the possibility that increased roughness and surface area enhanced neuron polarization for the PPy microchannels.
Figure 7.7. Phase-contrast, fluorescence and SEM photomicrographs, respectively, of hippocampal neurons on PPy. A) - C) Cells cultured on 2 µm wide and 200 nm deep PPy microchannels; D) - F) cells cultured on unmodified PPy. The green labeling (Alexa 488) corresponds to Tau-1 (axonal marker) immunostaining. Cells polarized more readily on microchannels than on unmodified PPy. Scale bar = 20 µm (A,B,D,E), 5 µm (C,F).
Others have also observed the effect of material topography on neuron polarization. Dowell-Mesfin et al. showed that silicon pillars of 2 μm width increased the number of cells in stage 3 after 24 h in culture [39], but quantitative measurements were not reported. More recently, Foley et al. showed increased neuritogenesis on nano-ridges in PC12 cells with low levels of NGF [40], and Ahmed et al. reported an increase in neurite generation in several neuronal types cultured on nanofibers [41]. These responses to topography are thought to be transduced by tension generated within the cytoskeleton and the redistribution of focal adhesion complexes (FAC) as a result of the patterns [reviewed in 42]. Additionally, the cytoskeleton is directly connected to the nuclear membrane that ultimately alters nuclear morphology, which has been hypothesized to be connected to changes in gene expression [42,43].

Axonal length of neurons was also investigated. Figure 7.8B shows that PPy microtopography did not result in significant increase in axon length from hippocampal cells when compared to cells cultured on unmodified PPy (P = 0.13 for 2 μm microchannels; P = 0.92 for 1 μm microchannels). We previously found this same trend on PDMS microchannels, as illustrated in Figure 7.8B. These results suggest that surface topographical features have a more dramatic effect on axon initiation mechanisms (i.e., polarization), but these effects become negligible once the axon is established and undergoes elongation. In contrast, we reported in Chapter 5 that ligands such as NGF have a stronger effect on axon elongation than topography. This trend suggests that physical guidance provided by PPy microchannels could be combined with chemical cues such as immobilized NGF to further optimize axon outgrowth.

Axon orientation was analyzed by determining the angle of growth with respect to the direction of the microchannels. Figure 7.9 shows a tendency for axons to grow either in a perpendicular (90°) or parallel orientation (0° or 180°)
with respect to the microchannels. However, this trend is not as pronounced compared to PDMS microchannels with the same dimensions. We hypothesize that less well-defined PPy microchannel morphology and geometry compared to PDMS resulted in lower orientation bias in the cells. Although we did not explore additional dimensions for cell studies, increased axon alignment is expected with increasing depth and gaps between microchannels.

![Figure 7.8](image)

**Figure 7.8.** Quantitative analysis of neuron polarization and axon length. A) Fraction of neurons with defined axons after 20 h in culture (n = 5 experiments with ~100 neurons analyzed per experiment) on PPy or PDMS substrates; B) Axon length of polarized cells (n > 100 axons) on PPy or PDMS substrates. The error bars correspond to SEM. Topography increased polarization but not axon growth. *,# Statistically significant differences from unmodified PPy/PDMS and 1 μm microchannels, respectively, using t-test with P< 0.05.
7.4 Conclusions

We have investigated neuron polarization, axon length and orientation on microstructured PPy. For this, 1 and 2 \( \mu \)m wide microchannels were patterned on ITO-coated slides using e-beam lithography and electropolymerization. We found that PPy microchannel morphology depended strongly both on the e-beam writing and the electropolymerization conditions, producing deeper, less-defined and rougher structures for higher e-beam writing currents, higher electropolymerization currents, higher pyrrole / dopant concentration and increasing polymerization time. Higher percentage of neurons cultured on patterned PPy polarized more readily, meaning that more cells had defined axons after 20 h in culture when compared to unmodified PPy substrates (two-fold increase). The effect of topography on axon length was found to be negligible, which is consistent with data on PDMS microchannels. These PPy microchannels could be further modified with immobilized NGF as described in Chapter 6, to take full advantage of multiple cues to stimulate cells. This modified PPy could be potentially applied in neural tissue engineering applications such as neural probes and nerve conduits.
Figure 7.9. Quantitative analysis of axon angle. Histograms for axon angle distribution on A) unmodified PPy/PDMS substrates; B) 1 µm PPy/PDMS microchannels; C) 2 µm PPy/PDMS microchannels. Angle was measured as illustrated in inset. Neurons extended axons in parallel or perpendicular orientations to the microchannels, but the trend on PPy microchannels was less defined than the trend on PDMS microchannels.
7.5 References


Chapter 8
Competitive Responses: Chemical Ligands versus Topography

Introduction

Neuron polarization produces the characteristic morphology of neurons with a single axon and multiple dendrites. We investigated in Chapter 5 and 7 the effect of extracellular cues on polarization of hippocampal neurons. These studies were focused on individual or combined stimuli that were homogenously presented on the substrate surface. This chapter describes the effect of “local” simultaneous environmental signals in the orientation of polarization, a more complex scenario. In particular, we analyzed the competitive responses between local stimulation to individual neurites with chemical ligands, including immobilized nerve growth factor (NGF) and laminin, and physical cues mediated by surface topography (i.e., microchannels).

8.1 Background and Motivation

Neuron polarization is defined as the establishment of a single axon (i.e., axogenesis) and multiple dendrites [1]. This particular neuronal morphology is critical for many cell processes, including connectivity with target cells and synapses [2]. Understanding polarization and neuronal behavior benefits both embryological development studies and regenerative strategies, including cell transplantation therapies for neurodegenerative diseases such as Parkinson’s and Alzheimer diseases [3,4]. For cell transplantation applications, the environment where stem cells are grown in vitro and transplanted into the injured brain is important, being essential to provide the most effective stimuli to control stem cell differentiation into neurons, cell migration and connectivity [5]. In light of
this need, studies on how extracellular cues can be designed and modified in matrices to be implemented as “artificial niches” that support cell therapy in the brain are essential [5]. Lutolf and Hubbell have emphasized the role of synthetic biomaterials as “instructive extracellular microenvironments” that can actively participate in determining cell fate in tissue engineering applications [6]. Along these lines, materials that can modulate neuronal behavior, including polarization, will be an important contribution for the creation of effective regenerative therapies.

Another important application for understanding neuron polarization is the creation of artificial neural networks. In this particular field, neurons are geometrically confined into patterns that allow the formation of a well-defined neural architecture for the control and study of synapses and neuronal signaling [7]. As part of the creation of these networks, the control over polarity and orientation of axons is essential for the understanding and reproducibility of the network characteristics. With this purpose, studies on cues such as patterning of adhesive molecules have been investigated to control polarization of neurons and axon orientation [7,8].

Although polarization has been extensively studied, it is still not completely well-understood how intracellular and extracellular cues modulate this process. Embryonic hippocampal neurons are the most characterized in vitro model to investigate polarization mechanisms [9]. Early work by Banker and colleagues showed that these cells undergo polarization during the first 24-48 hours in culture [1,9] following 5 stages: In stage 1, neurons are unpolarized; in stage 2, there is an outgrowth of minor processes or neurites; stage 3 cells form a single axon from the growing neurites (i.e., polarized neuron). Finally, in stage 4 cells, dendrites begin growing and developing, and stage 5 neurons are fully-mature neurons [9].
Neuronal polarity is determined by both a positive feedback loop at the growth cone of the future axon that enhances growth, and a negative feedback loop that propagates throughout the neuron to inhibit the other neurites from growing further [10,11]. Some key molecules in the positive feedback loop are Cdc42, PIP3, GSK-3β and PAR-3 [12-15], which ultimately contribute to the increased actin polymerization and microtubule assembly that produces accelerated growth of the established axon. These molecules could be modulated in vivo by extracellular stimuli [16], including biochemical cues such as laminin [17] and NGF [18], and physical cues such as surface topography [19]. We described the effect of immobilized NGF and topographical features on polarization in Chapter 5.

An even more complex scenario is the “local” stimulation of individual neurites before polarization. For example, laminin induces the formation of an axon when a single neurite of a stage 2 cell gets in contact with this protein [13,21]. In a similar way, tension exerted by a micropipette on an individual neurite induces axon formation [22]. These studies demonstrated that localized extracellular stimuli play a key role on determining polarization. Here, we have further explored localized stimulation of neurites by presenting two simultaneous, competitive and confined cues to individual hippocampal neurons. For these studies, immobilized NGF, laminin and surface microtopography in the form of microchannels were analyzed. NGF and laminin are chemical stimuli mediated by receptor-ligand interactions, whereas topographical cues are physical signals mediated by multiple events related to cytoskeleton tension [23].

The local effect of the single cues was first analyzed to find that all three stimuli (i.e., microchannels, immobilized NGF and laminin) predominantly direct the formation of an axon when the cues are individually presented to the cells. Neuron polarization was subsequently studied when topography and either NGF
or laminin were simultaneously presented. We found that topographical features are preferred over chemical ligands for axon formation (70% of axons chose topography), suggesting that contact guidance mechanisms exert a stronger effect on polarization. These results contribute to the understanding of neuronal behavior on synthetic materials that could be potentially used in regenerative medicine and artificial neural networks, and demonstrates for the first time integration of simultaneous physical and chemical cues in cultured neurons.

8.2 Materials and Methods

8.2.1 Microfabrication of Channels

Microchannels 1 and 2 µm wide and 400 nm deep were created on PDMS using soft lithography techniques as described in Chapter 4. Briefly, the procedure had three steps: 1) fabrication of a mask with the desired patterns, 2) fabrication of a silicon master, and 3) replica molding of PDMS.

The mask was created with electron beam (E-beam) lithography. Patterns were written on silicon wafers with a SiO₂ layer, which were spin-coated with 4% poly(methyl methacrylate) PMMA resist in chlorobenzene (Microchem). Microchannels of 1 or 2 µm were written on the PMMA resist using E-beam (Raith-50 and XL-30 SEM, LaB₆ source) with an area dose of 220 µAs/cm² and beam current of 0.2 nA.

After resist development, a thermal evaporator (Denton) was used to deposit a 100 nm chromium (R.D. Mathis) film and lift-off with acetone was performed subsequently. Reactive Ion Etching (RIE) of the SiO₂ layer was performed in an etcher (Plasma Technology) with a mixture of CHF₃ and oxygen. The final master was silanized with tridecafluoro-1,1,2,2-(tetrahydrooctyl) trichlorosilane (Gelest), and subsequently used for replica molding of PDMS (Sylgard 184, Dow Corning).
All PDMS substrates with microchannels were sonicated in 70% ethanol for 10 minutes, UV-sterilized for 1 h, and placed inside either sterile 3 cm or 10 cm (cell micropositioning) Petri dishes. Substrates were coated by incubation with a solution of polyallylamine in water (7 mg/mL) overnight, and washed twice with distilled-deionized water.

8.2.2 Fabrication of Competition Scheme

A competition scheme was created by immobilizing ligands next to the surface microchannels in PDMS (see Figure 8.1 for schematic of the process). A separate PDMS well was fabricated by cutting a rectangle (8 mm X 3 mm) in the center of a PDMS film (1.3 mm X 7 mm) with a razor blade, which was subsequently sterilized with ethanol and UV exposure. This well was placed on top of a PDMS substrate with microchannels, using fine tweezers and a light microscope inside a horizontal hood to maintain sterility. The inside rectangle edge of the well was positioned 10 - 30 \( \mu \)m away from the edge of the microchannels, and pressed down to tightly seal the well (Step 1 in Figure 8.1).

After the PDMS well was positioned parallel to the microchannels, immobilization of NGF (Step 2A in Figure 8.1), or laminin coating (Step 2B) was performed exclusively inside the well. NGF immobilization procedure is carefully explained in the following paragraphs. Conventional laminin coating was performed by overnight incubation at 4°C with a 10 \( \mu \)g/mL laminin (Trevigen) solution, and washing with distilled-deionized (DDI) water. After either NGF immobilization or laminin coating, the PDMS well was removed from the substrate to obtain the competition scheme (Step 3 in Figure 8.1).
8.2.3 NGF-FITC Conjugation

NGF was conjugated to fluorescein for detection and characterization of the immobilization procedure as described in Chapters 5 and 6. Briefly, 100 µL of NGF 2.5S (Promega, 100 µg/ml) was reacted with 10 µL of fluorescein isothiocyanate in dimethyl sulfoxide (FITC, 12 mg/mL) (Molecular Probes), and separated with size exclusion chromatography columns (Biorad, exclusion limit 6,000 Da). NGF-FITC was only used for quantification and visualization purposes. A calibration curve for NGF characterization was obtained and used for determining NGF surface concentration as in Chapters 5 and 6.

8.2.4 NGF Immobilization on Competitive Geometry

NGF photochemical fixation was performed using a phenyl-azido group as described in Chapter 5. Briefly, the procedure consisted of three main steps: 1) preparation of N-4-(azidobenzoyloxy)succinimide according to a previously published procedure [42]; 2) polyallylamine (PAA) conjugation to N-4-(azidobenzoyloxy)succinimide, and 3) fixation of NGF using the modified polyallylamine. After synthesis of N-4-(azidobenzoyloxy)succinimide, a solution of 15 mg of polyallylamine (Aldrich) in 10 mL of phosphate buffered solution (PBS, pH = 7.4) was added to a solution of 13 mg of N-4-(azidobenzoyloxy)succinimide in 5 mL of N,N-dimethylformamide and stirred for 24 h at 4°C. The solution was ultrafiltered (Millipore, 10,000 Da) and washed three more times by adding 10 mL of DDI water and ultrafiltered again to finally obtain a volume of ~300 µL of photosensitive polyallylamine (PAA-Azido). The conjugate was diluted in DDI water to obtain a final volume of 2.4 mL (1:8).

50 µL of PAA-Azido solution were cast inside the positioned well on a PDMS substrate with microchannels, air dried and exposed with a UV lamp (Blak-Ray, 22 mW/cm², λ_max = 365 nm) for 15 seconds followed by three washes.
with 0.01 M HCl and two washes with PBS. This step was followed by casting a second layer of the photosensitive polyallylamine (50 µL) and a superimposed final layer of NGF (for cell culture) or NGF-FITC (for quantification) (1 µg in 50 µL of PBS). Finally, the substrate was exposed to UV light for 15 seconds and washed six times with PBS to remove unreacted protein and two more times with DDI water. The superimposed well was then removed, the complete PDMS substrate washed two more times with DDI water and air-dried.

8.2.5 Hippocampal Cell Culture and Immunochemistry

Embryonic rat hippocampal cells (E18) were isolated from commercially-obtained hippocampus tissue (BrainBits). The hippocampi were incubated with papain (Worthington) in Hibernate E medium (Brainbits) (4 mg/mL) at 30°C for 20 minutes, followed by physical trituration with a fire-polished pasteur pipette. Cells were counted and plated randomly (7.5 x 10^3 cells/cm^2) or using a micropositioner on the prepared PDMS substrates with the competition scheme, and cultured with Neurobasal Medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), L-glutamine (Fisher, 0.5 mM), L-glutamic acid (Sigma, 25 µM) and 1% antibiotic-antimycotic (Sigma, 10,000 units/mL of penicillin, 10 mg/mL of streptomycin and 25 µg/mL of amphotericin). After 44 h in culture at 37°C and 5% CO2, cells were fixed with 4% paraformaldehyde (Sigma), 4% sucrose (Fisher) in PBS for 20 minutes, followed by permeabilization for 20 minutes with 0.1% Triton-X100 (Sigma) in 2% bovine serum albumin (BSA) (Jackson ImmunoResearch) in PBS, and blocking for 1 h at 37°C with 2% BSA-PBS. Samples were incubated with antibodies for Tau-1 (axonal marker) (Chemicon, 1:200), NGF (Abcam, 1:200) or laminin (Sigma, 1:200) in 2% BSA-PBS overnight at 4°C, followed by incubation with secondary antibodies (Alexa 488-conjugated, Molecular Probes, and TRITC-conjugated, Sigma), for 1 h at 37°C.
8.2.6 Cell Micropositioning

For single-stimulus experiments, hippocampal neurons were randomly placed on substrates containing the stimuli (Figure 8.1, step 4), and only cells in the vicinity of the stimulus were analyzed (see following sections for criteria). For competitive experiments with two simultaneous cues, micropositioning techniques were used to place cells precisely between the stimuli. For this process, thin micropipettes were obtained by pulling glass capillaries (World Precision Instruments) with a vertical pull type puller (Narishige). The pulled-micropipette was connected to a pneumatic microinjector (Narishigue Group) and tightened to an XYZ movable micromanipulator (Narishigue). This setup was mounted on a reflectance upright microscope (Olympus BX51WI) located inside a horizontal laminar airflow workstation to guarantee sterility in the procedure.

PDMS substrates with competition cues were placed inside 10 cm sterile Petri dishes. After cell trituration, neurons in culture medium (2 x 10⁵ cells/mL) were added on the periphery of the PDMS substrate and allowed to settle for 15 minutes. Before performing the micropositioning, additional medium was added to the Petri dish to cover the PDMS surface. A single neuron was identified on the Petri dish (outside the PDMS), aspirated with the micropipette by creating suction with the injector, moved with the micromanipulator, and positioned between the cues on the PDMS substrate by releasing the cell. The same procedure was repeated for approximately 6 - 8 cells on a single substrate in about 20 - 30 minutes, and 4 - 6 different samples were consecutively done per day. For each condition, experiments were performed on at least three different days. After micropositioning, Petri dishes were incubated at 37°C and 5% CO₂.
8.2.7 Fluorescence Microscopy

NGF-FITC immobilization, cell polarization and axon extension were analyzed using an inverted phase contrast and fluorescence microscope (IX-70, Olympus). Images from the microscope were acquired using a color CCD video camera (Optronics MagnaFire, model S60800) and analyzed using Adobe Photoshop and Image J (NIH).

8.2.8 Polarization and Competition Analysis

Based on published criteria, a hippocampal cell was defined as polarized (stage 3) when one of its neurites (i.e., the axon) was at least twice as long as the other neurites and it stained positively for Tau-1 [9,12,13,15]. Only cells that were already polarized after 44 h in culture were analyzed for competition. For random cell placing, a single neuron next to either microchannels or chemical ligands was analyzed for competition when it was within one cell body from the stimulus, and when it was separated from other neighbor neurons by three cell bodies in distance. At least 20 cells were analyzed per stimulus. For competition analysis, only micropositioned cells that were within one cell body distance from both cues were analyzed. A neuron was defined as polarized toward a specific side when a single axon was growing on one of the presented stimuli (i.e., on top of microchannels, NGF or laminin) after 48 h in culture. A total of 10 - 20 micropositioned cells were analyzed per competition condition, which is consistent with other previous studies on responses of individual neurons or growth cones [25,26].

Distributions of axons for all conditions were tested against a 50% equal probability distribution using a $\chi^2$ test, to demonstrate that cells were preferentially choosing one of the two possible stimuli. In addition, distributions of axons in competition geometries between physical and chemical cues were
tested against the two distributions of the individual stimuli using a $\chi^2$ test, to show a significant difference in axon formation.

8.2.9 **Scanning Electron Microscopy (SEM)**

Microchannels and cells on PDMS were analyzed with a LEO 1530 scanning electron microscope. For imaging neurons, cells on PDMS substrates were fixed with 4% paraformaldehyde (Aldrich) and 4% sucrose in PBS for 20 minutes, and dehydrated with increasing concentration of ethanol (30% - 100%) for a total time of 2 h, followed by 5 minutes exposure to hexamethyl-disilazane (Sigma). After drying, samples were coated with a gold layer for SEM measurement and imaged with a typical acceleration voltage of 1 kV.

8.3 **Results and Discussion**

8.3.1 **Fabrication and Characterization of Competitive Scheme**

In Chapter 5, immobilized NGF was shown to be active, producing similar responses in hippocampal neurons when compared to controls with soluble NGF. In addition, microchannels of 1 and 2 $\mu$m in width significantly increased the number of stage 3 cells. These results suggest that both immobilized NGF and microtopography significantly influence neuron polarization. Here, we studied how polarization is regulated when both cues are presented to the neuron simultaneously and independently.

To study this novel competition scheme that consisted of an area with microchannels separated from an area with either immobilized NGF or laminin was created. After the fabrication of microchannels on PDMS using conventional microfabrication techniques, a PDMS well was positioned next to the
microchannels area. Next, NGF was immobilized on the area enclosed by the superimposed well as previously reported [20], using aryl-azido conjugates and UV exposure (Figure 8.1, Step 2A). Similarly, laminin was adsorbed for different competition experiments with a concentration already reported to affect polarization [17] (Figure 8.1, Step 2B).

Figure 8.2 shows the results for the creation of the competition scheme. NGF was fluorescently labeled with FITC for visualization of the area covered by the tethered protein. NGF-FITC was exclusively immobilized in an area adjacent to the microchannels and separated by a distance of approximately 20 µm, as illustrated in Figure 8.2C. The surface concentration of immobilized NGF was found to be 0.11 ng/mm², which was analyzed from the fluorescence intensity of immobilized NGF-FITC. Laminin was adsorbed to create similar competitive schemes as illustrated in Figure 8.5.

8.3.2 Neuron Polarization at Interfaces with Individual Stimuli

The first step in the process of testing competition responses in cells was to analyze if the individual stimuli could control the direction of polarization. For this analysis, neurons located on the edge of the particular cue, either next to microchannels or next to the interface of the immobilized NGF or laminin areas, were analyzed.

8.3.2.1 Polarization Orientation with Physical Guidance Cues

Figure 8.3 summarizes the data for the effect of topographical features on polarization. Both 1 µm and 2 µm microchannels effectively influenced axon formation. Neurons preferentially defined an axon toward the micropatterned area rather than on the smooth (i.e., no microchannels) area. This is the first time
contact guidance cues are found to influence axogenesis at micropatterned interfaces. From the quantitative data, 1 μm features have a larger effect with 80% of the cells polarizing on the topography side (χ²=8.9, P=0.003 for parallel microchannels; χ²=6.9, P=0.008 for perpendicular microchannels). Microchannels of 2 μm predominantly induce 65 - 75% of the cells to define an axon on the micropatterned area (χ²=9, P=0.003 for parallel microchannels; χ²=5.6, P=0.02 for perpendicular microchannels). There is a significant difference between axon distributions on parallel and perpendicular microchannels (χ²=36.2, P<0.001 for 2 μm microchannels; χ²=21.5, P<0.001 for 2 μm microchannels). Overall, 75% of neurons preferred to polarize on micropatterns (n= 114, χ²=29.5, P<0.001).

The only previously reported effect of localized physical cues on polarization involved the use of micropipettes to create tension by pulling single neurites in stage 2 neurons [22]. This investigation showed that an individual neurite of a stage 2 neuron could be towed and induced to become the axon. The authors proposed that tension in the growth cone induces axon definition, which is related with increased traction and growth.
Figure 8.1. Fabrication of competitive scheme between physical and chemical cues for neuron polarization. Microchannels are fabricated in PDMS using microlithographic techniques. After this, a PDMS well is aligned along the edge of microchannels under the microscope (1). NGF immobilization using aryl-azido photolinkers (2A) or laminin coating (2B) is performed inside the PDMS well. After extensive washing the PDMS well is removed (3), which finally renders an area modified with chemical ligands that is parallel to microchannels. Hippocampal neurons are subsequently cultured on the modified PDMS substrates for competition studies (4). Random cell culture is used to test the effect of an individual stimulus effect on polarization (dotted ovals show cells analyzed). Micropositioning is used when testing competition between two cues.
Topography is a cell stimulus that is believed to be intracellularly transduced by tension in the cytoskeleton and the cell membrane [23]. Tension created by surface topographical features is associated with alteration of nuclear morphology and the upregulation of multiple genes [27]. In addition, topography effects are also associated with the opening of calcium channels [28], which could initiate multiple intracellular pathways [3], the redistribution of focal adhesion complexes and integrins, and activation of tyrosine kinases [23,29]. Based on this evidence, we hypothesize that topographical features could locally stimulate positive feedback loops for growth by exerting cytoskeleton tension. In particular, the importance of actin dynamics in axon formation has been studied and found to be definitive in polarization of neurons [26]. Therefore, the effect of topographical features on actin polarization in a single neurite could initiate the formation of the axon.
Another possible effect of topography could be the formation of an uneven structure in the cell soma as a consequence of the irregularity of the surface. Previous studies using AFM showed that higher or bulkier regions of the soma are related to the initial formation of neurites [30]. Based on this aspect, microchannels could possibly affect the structure of the cell soma and induce formation of the axon as a consequence of this unevenness.

8.3.2.2 Polarization Orientation with Chemical Guidance Cues

Figure 8.4 illustrates the data for the effect of chemical ligands, both immobilized NGF and laminin. The interface of the modified areas was only visible with fluorescence microscopy after immunostaining. Similar to the physical cues, chemical cues primarily induced the formation of axons on the modified areas containing the protein. 71% and 65% of neurons preferred to extend axons on immobilized NGF and laminin, respectively, compared to unmodified surfaces ($\chi^2=15.1$, $P<0.001$ for immobilized NGF; $\chi^2=5.9$, $P=0.01$ for laminin). Overall, 69% of neurons statistically preferred to polarize on chemical cues ($n=141$, $\chi^2=20.7$, $P<0.001$).

Laminin is known to locally induce axogenesis [13,21]. Esch et al. fabricated substrates with stripes of either laminin or neuron-glia cell adhesion molecule (NgCAM) [21]. The authors found that neurites in contact with either of these molecules became axons, with 80% of axons being formed on laminin stripes. In this same study, increased growth of the neurite in contact with laminin was shown to occur almost immediately after the first contact with laminin, inducing the formation of the axon and simultaneously decreasing growth of the other neurites.
Figure 8.3. Effect of physical stimuli on neuron polarization. Hippocampal neurons that were adjacent to the interface of microchannels were analyzed for the direction of axon formation. A) and B) Phase-contrast and fluorescence (Tau-1 labeling) images, respectively, of a neuron next to 2 µm microchannels. Axon is established on the surface with topographical features. Scale bar = 25 µm; C) Quantitative analysis of polarization of neurons next to microchannels. The X-axis represents the dimensions and orientation of the microchannels (perpendicular and parallel definitions are illustrated in Figure 8.1). The Y-axis represents the percentage of cells that established axons on either the microchannels (physical) or smooth areas (no stimulus). The majority of neurons polarized toward the micropatterned area (P=0.003, 0.02, 0.008,0.003 for each dimension from left to right in the X-axis, respectively).
A more recent study corroborated the same trend of localized growth, and demonstrated the accumulation of PIP$_3$ in the neurite in contact with laminin stripes [13]. This accumulation occurs 5-20 minutes after the first contact, and increases the growth rate from $\sim$1 $\mu$m/h to $\sim$50 $\mu$m/h. This investigation proposed that the positive feedback for the formation of the axon is activated by PI 3 kinase and PIP$_3$, which ultimately activates the GTPases Rac and Cdc42 that enhance actin polymerization.

NGF has not been investigated before for inducing localized axon formation. We reported the retained activity and similar responses of immobilized NGF when compared to soluble NGF [20]. In addition, the increase in polarization and axon growth was demonstrated when the growth factor was uniformly immobilized on the substrate. In the present study, the immobilization of NGF was used as a tool to localize it on the substrate surface and analyze the orientation of axon formation. We found that immobilized NGF was capable of inducing the formation of an axon in the direction of the area containing the protein. These results were comparable to previous studies [13,21] and our own results with laminin.

NGF signaling in hippocampal neurons is primarily mediated by the p75$^{NTR}$ receptor [18]. In this pathway, the binding of NGF to this receptor produces the formation of ceramide, which accelerates polarization of hippocampal neurons [18]. The molecules downstream of ceramide formation that produce axon formation have not been thoroughly investigated, but pathways that connect to jun kinase have been proposed [18, 31]. We hypothesize that the ceramide cascade leading to increased polarization with NGF can be localized to the neurites only in contact with the immobilized growth factor, producing the subsequent formation of axons.
Figure 8.4. Effect of chemical stimuli on orientation of neuron polarization. Hippocampal neurons that were adjacent to the interface of immobilized NGF or laminin were analyzed for the direction of axon formation. A) Phase-contrast image, B) fluorescence image (Tau-1 labeling) and C) overlay of Tau-1 and NGF labeling images of a neuron next to immobilized NGF. Axon is established on the surface with immobilized NGF. Scale bar = 25 µm; D) Quantitative analysis of polarization of neurons next to chemical stimuli. The X-axis represents the type of ligand at the interface. The Y-axis represents the percentage of cells that established axons on either the chemical ligand or unmodified areas. The majority of neurons polarized toward the chemical stimulus (P<0.001 for immobilized NGF; P=0.01 for laminin).

A recent publication investigated the effect of NGF and TrkA signaling in the polarization of hippocampal neurons [32]. This paper investigated in detail the segregation of the plasma membrane ganglioside sialidase (PMGS) in neurites that transform into axons. The authors found that this molecule was primarily segregated to the neurite that becomes the axon, before actually being polarized. In addition, the segregated PMGS was closely related to phosphorylated TrkA receptors in the developing neurite, which was correlated to NGF binding to this
receptor. In light of this, immobilized NGF could locally activate TrkA, cross-phosphorylate tyrosines and initiate cascades inducing the segregation of molecules for polarization such as PMGS. PMGS induces inactivation of RhoA by modulating PI 3 K and Rac1, which are mediators of the positive feedback loop that leads to increased growth rate of the developing axon.

8.3.3 Neuron Polarization with Competitive Physical and Chemical Stimuli

After analyzing the individual effect on polarization direction of individual stimuli, competition between chemical and physical cues was investigated. We studied three different competition combinations with perpendicular microchannels as shown in Figure 8.5. We found that physical guidance cues were statistically preferred by polarizing neurons over chemical guidance for two cases. 1 μm microchannels induced 74% of axons to grow on the micropatterned area when simultaneously presented with immobilized NGF ($\chi^2=5.55$, $P=0.02$). 2 μm microchannels had 64% ($\chi^2=1.63$, $P=0.2$, not statistically different from 50% equal distribution) and 69% ($\chi^2=5$, $P=0.03$) of axons on the micropatterned area when simultaneously presented with immobilized NGF and laminin, respectively. Overall, physical cues were statistically preferred (70%) over chemical cues for axon formation ($n=69, \chi^2=11.4$, $P<0.001$).

Axon distributions on microchannels in the presence of competing chemical cues were statistically different from the distributions for the individual stimuli using a $\chi^2$ test ($P < 0.001$ for all three combinations when compared to individual microchannels or chemical ligands). These results suggest that integration of multiple independent extracellular signals in the neuron is possible, which ultimately determines the decision for polarization. In general, physical cues were statistically preferred over chemical ligands, which correlates with our previous results described in Chapter 5.
Although both immobilized NGF and laminin can initiate positive feedback loops probably by PI 3 kinase activation [13,32], the results presented here indicate that this specific activation is not sufficient in the presence of the cascades activated by topography. We hypothesize this behavior could be a consequence of an overall activation of multiple cascades by topography, which could include various simultaneous receptors and molecules such as integrins, tyrosine kinases in focal adhesions, calcium channels and actin [23,29]. The nonspecificity of the topography transduction could trigger various intracellular pathways, producing a more effective feedback mechanism than the limited cascades derived from the ligands. In addition, 1 \( \mu \)m microchannels are more effective in inducing polarization. This particular dimension has previously been correlated with perpendicular alignment of axons [20,28,33]. Nagata et al. suggested that 1 \( \mu \)m structures mimic tightly-aligned neurite bundles provided by other cells in the body [33], which could represent an important stimulus \textit{in vivo}.

8.3.4 \textit{Competition Between a Combination of Physical and Chemical Stimuli vs. Physical Stimulus}

The final competition analysis was chosen based on the fact that physical cues were found to be stronger than chemical ligands. We investigated if a combination of physical and chemical cues could be stronger than physical cues alone. For this, 2 \( \mu \)m microchannels were presented to neurons on both sides, but one of the microchannel areas was also modified with immobilized NGF (\textbf{Figure 8.6A} and 8.6B). In addition to this scheme, a control geometry was also analyzed when both sides only had microchannels, which theoretically would give equal chances for polarization (i.e., 50 %).
Figure 8.5. Competition between chemical and physical stimuli on neuron polarization. Hippocampal neurons were micropositioned between immobilized NGF or laminin and microchannels, and analyzed for the direction of axon formation. A) Overlay of phase-contrast and fluorescence (green labeling for Tau-1, red labeling for laminin) images of neuron between 2 µm microchannels and laminin. Axon was established on the surface with microchannels. Scale bar = 25 µm; B) SEM image of neuron in A (pseudo colored for visualization). Scale bar = 12 µm; C) Quantitative analysis of polarization of neurons between chemical and physical stimuli. The X-axis represents the specific competition pair. The Y-axis represents the percentage of cells that establish axons on either the chemical or physical stimuli. The majority of neurons polarized toward the physical stimulus (P=0.2, 0.02 and 0.03 for each combination from left to right in the X-axis, respectively).
Figure 8.6C shows that for the control system, each side was chosen 46% and 54% of the times, respectively, which is statistically equal to the 50% expected (P=1). Furthermore, when one of the areas was combined with immobilized NGF, that specific side was chosen 61% of the times but this value was not significantly different from a 50% equal distribution ($\chi^2=0.88$, P=0.35). This result suggests that topography dominates the response. However, chemical ligands could play an important role when combined with physical cues for both polarization and subsequent growth, which could be the in vivo situation for arrangements of cells (e.g., glia) that express or secrete specific ligands [34].

8.4 Conclusions

We investigated a novel aspect of neural behavior: polarization in response to competitive stimuli. In particular, we analyzed competition between chemical (i.e., immobilized NGF and laminin) and physical (i.e., topography) cues. We first analyzed the effect of the individual stimuli, finding that both topography and chemical ligands effectively influence axon formation when presented independently (75% and 69% of axons grew on physical and chemical cues, respectively, compared to unmodified surfaces). When both chemical and physical cues were presented simultaneously to the cells, physical cues were preferred 70% of the times over the chemical signals. When topography was combined with immobilized NGF and tested against topography alone, axons preferred to grow on the combination side 61% of the times. The results derived from this study contribute to the understanding of neuronal behavior on artificial substrates, which is applicable to the creation of artificial niches for neuron transplantation on the brain, neural tissue engineering applications and control of polarity in neural networks.
Figure 8.6. Competition between combined microchannels and immobilized NGF and microchannels alone. Hippocampal neurons were micropositioned between microchannels with immobilized NGF and microchannels only, and analyzed for the direction of axon formation. A) Overlay of phase-contrast and fluorescence (green labeling for Tau-1; red labeling for NGF) images of neuron between 2 µm microchannels and combined 2 µm microchannels with immobilized NGF. Axon was established on the surface with the combined cues. Scale bar = 25 µm; B) SEM image of neuron in A (pseudo colored for visualization). Scale bar = 12 µm; C) Quantitative analysis of polarization of neurons between combined stimuli and microchannels only. The X-axis represents the specific competition pair. The Y-axis represents the percentage of cells that established axons on either the combination or microchannels only (P=0.35 and 1 for combination and microchannels only, respectively).
8.5 References


Chapter 9
Conclusions and Recommendations

9.1 Summary of Dissertation

The creation of bioactive materials is a critical aspect for tissue engineering applications. Lutolf and Hubbell have emphasized the role of synthetic biomaterials as “instructive extracellular microenvironments” that can actively participate in determining cell fate [1]. Biomaterials should not only provide support for cell growth but also actively interact with cells to promote specific behaviors such as proliferation, migration or differentiation [1,2]. In particular, neural tissue engineering applications such as nerve conduits for nerve regeneration [3], neural prosthetics such as retinal and cochlear implants [4], and the creation of artificial neural networks [5] would be highly benefited from the understanding of how to accurately modulate neuronal behavior on artificial substrates.

With the ultimate goal of creating materials that stimulate neuronal behavior, this dissertation focused on the modification of material surfaces that provide effective cues for influencing neuron polarization and axon outgrowth. There are many extracellular stimuli previously investigated to influence neuron responses, including chemical cues (growth factors, ECM components, etc), physical cues (topography, tension, etc), interaction with glial cells and electrical stimulation (summarized in Chapter 2). This investigation studied three of these cues (reviewed in Chapter 3): 1) chemical guidance by growth factors, in particular nerve growth factor (NGF), which was immobilized on material surfaces; 2) physical guidance provided by surface topography; and 3) electrical stimulation by using the electrically-conducting polymer, polypyrrole. More
importantly, combinatorial and competitive stimuli were investigated to further enhance neuron responses and to better understand decision-making processes.

A combination of physical guidance and chemical guidance cues was first analyzed. For this strategy, microchannels were fabricated in poly(dimethyl siloxane) (PDMS) (Chapter 4), which were subsequently modified by immobilizing NGF on the surface (Chapter 5). The microchannels provided a physical stimulus, whereas the immobilized NGF provided a chemically-bioactive surface.

Embryonic hippocampal neurons were used to investigate the impact of these surface cues on polarization (i.e., axon initiation or axogenesis) and overall axon length. We found that topography had a more pronounced effect on polarization (68% increase over controls) compared to immobilized NGF (0.1 ng/mm²) (27% increase). However, the effect of NGF was negligible when both types of stimuli were simultaneously presented on the biomaterial surface. On the contrary, the most evident effect for overall axon length was for the immobilized growth factor (10% increase in axon length with respect to controls) whereas there was no effect in general for the microtopography. More importantly, when the two surface stimuli were presented in combination, a synergistic increase in axon length was detected (25% increase with respect to controls).

A second combination consisting of chemical guidance by immobilized NGF and electrical stimulation was investigated (Chapter 6). For this approach, NGF was immobilized on the surface of the electrically conducting polymer polypyrrole (PPy). Three different NGF surface concentrations were obtained (0.21-0.98 ng/mm²) and similar levels of neurite extension in PC12 cells were observed on immobilized NGF as with soluble NGF. Additionally, electrical
stimulation experiments were conducted with the modified polymer and revealed an increase of 50% in neurite outgrowth in PC12 cells compared to experiments without electrical stimulation.

Based on the results from the two previous combinations (i.e., topography plus immobilized NGF and PPy plus immobilized NGF), we hypothesized that a triple combination consisting of topography with immobilized NGF and electrical stimulation provided by PPy would further enhance neuron responses. The first step toward that combination was the creation of microchannels in PPy. These micropatterns were fabricated using electron beam lithography and electropolymerization (Chapter 7). A systematic analysis of parameters controlling PPy micropatterning was performed, finding that microchannel depth, roughness and morphology were highly dependent on e-beam writing current, polymerization current, pyrrole / dopant concentrations and polymerization time. Hippocampal neurons cultured on patterned PPy polarized faster on this modified material, having a two-fold increase in the number of cells with defined axons compared to cells cultured on unmodified PPy. These topographical features also had an effect on axon orientation but did not have a significant effect on overall axon length, which corroborates the previous data obtained on PDMS microchannels in Chapter 5.

Finally, we investigated a novel type of cell response where two stimuli were simultaneous but independent (Chapter 8). In particular, we analyzed for the first time the competitive responses between chemical ligands, including immobilized NGF, and physical cues mediated by surface topography (i.e., PDMS microchannels). First, the effect of individual stimuli was investigated by analyzing if axons were formed in the direction of the presented stimuli. The majority of axons were found to form on either microchannels (75%) or chemical ligands (69%) when these were independently presented to neurons. Secondly,
competition between physical and chemical signals was assessed by simultaneously presenting the cues to polarizing neurons. In this case, physical cues were preferred 68% of the times for polarization over chemical ligands, which suggests a stronger stimulation mechanism triggered by topography. Finally, topography was combined with immobilized NGF and tested in competition against topography alone, which showed that axons preferred to grow on the surface with combined stimuli side 61% of the times.

9.2 Conclusions

Immobilized NGF (0.1 ng/mm$^2$) produced similar responses (i.e., polarization and axon length) in hippocampal cells as soluble NGF (50 ng/mL), which allowed us to hypothesize the equivalence of intracellular pathways, even without endocytosis in the immobilized form. For PC12 cells, a surface NGF concentration of 0.98 ng/mm$^2$ produced similar neurite extension compared to soluble NGF after 2 days in culture. However, although immobilized NGF maintained the effect on neurite length after 10 days in culture, some detrimental effects were observed as a potential consequence of inhibition of endocytosis-dependent pathways.

Neuron polarization was analyzed and we concluded that surface topography more effectively influenced polarization than biochemical ligands such as NGF or laminin. In contrast, effects on axon length seemed to be opposite. Immobilized NGF and laminin had a greater increase, whereas topography did not have a significant effect. Furthermore, a synergistic enhancement for overall axon length was observed when both contact guidance and immobilized NGF were simultaneously presented on the surface, which could be a result of faster polarization from topography plus increased growth rate from NGF.
With respect to electrical stimulation using PPy-NGF, combination of chemical and electrical stimulation increased neurite length compared to controls without electrical stimulation. Cell adhesion was also improved as a consequence of the positively charged surface and the increase in roughness that resulted from the immobilization procedure.

PPy microchannel morphology was found to strongly depend both on the e-beam writing and the electropolymerization conditions, producing deeper, less-defined and rougher structures for higher e-beam writing currents, higher electropolymerization currents, higher pyrrole / dopant concentration and increasing polymerization time. Following the same trend found on PDMS microchannels, we concluded that PPy microtopography greatly influenced polarization, but the effect on axon length was negligible.

Finally, in the competition studies for orientation of polarization, we concluded that both topography and chemical ligands effectively influenced axon formation when presented independently. However, when these were simultaneously presented to the cells, physical cues were preferred the majority of the times for the formation of the axon.

Overall, physical or contact guidance cues seem to play a definitive role in neuron polarization as an initiation process. This was found both by the increase in the number of polarized cells that were cultured on topographical features, and by the preference of neurons to establish an axon in the direction of topography (i.e., in competition studies). In contrast, chemical ligands seem to play a major role in axon length, which is associated with an elongation process after axon formation. This is the first time the direct effects of these extracellular cues were analyzed with respect to the type and the time of the responses.
The studies presented in this dissertation have contributed to further knowledge regarding the modification of artificial substrates to control and modulate neuronal behavior. The results from this investigation can be applied in the design of better materials for nerve regeneration strategies, the modification of electrodes that interact with neurons in the body after transplantation of prosthetic devices, and for the control of neuronal mapping, architecture and connectivity in neural nets.

9.3 Future Directions

9.3.1 Triple Combination: Electrical, Chemical and Physical Stimulation

Chapter 7 described the first step toward triple stimulation including electrical stimulation with PPy, physical stimulation with microchannels, and chemical guidance with immobilized NGF. The studies on combinations described on Chapters 5 and 6 showed the potential to enhance neuron responses to artificial substrates by combining multiple stimuli. These results allowed us to hypothesize that a triple combination provided by a conducting polymer with physical and chemical guidance cues could further control and increase neuron behavior.

PPy microchannels have been fabricated and found to produce the same effect on polarization an axon length as PDMS microchannels. The following steps to study the possible enhancement by the triple combination are:

1) Culture hippocampal cells on PPy and analyze the effect of electrical stimulation on polarization and axon length. In Chapter 6, PC12 cells were initially used because these were previously analyzed in electrical stimulation experiments in the literature [6]. However, hippocampal neurons are an excellent
model for analyzing polarization and axon length with the potential to show more significant and relevant results than the ones obtained in chapter 6. This study would be very novel as no previous investigation of the effect of electrical stimulation on neuron polarization has been reported before.

Modification of the electrical stimulation protocol would be required because the time-frame for analyzing polarization in hippocampal cells would be of 24 h. A first possible protocol could be to plate cells after trituration, allow cell adhesion for 3 h, electrically stimulate for 2 h with 100 mV, and analyze at 24 h after plating.

2) Immobilize NGF on PPy microchannels (Figure 9.1) and corroborate the results obtained in chapter 5 (PDMS microchannels plus immobilized NGF) with this new modified material.

3) Perform electrical stimulation on hippocampal neurons cultured on PPy microchannels with immobilized NGF. This study would reveal if the triple combination provides any advantages over the dual duplications already analyzed in this dissertation.
Figure 9.1. Schematic of the NGF immobilization process on PPy microchannels. PAA is conjugated to an azido compound to produce PAA-azido. This conjugate is cast twice on PPy microchannels, followed by casting of NGF. UV light exposure promotes the formation of covalent bonds via the azido groups, immobilizing NGF to PPy microchannels.

9.3.2 Competition Analysis for Axon Guidance

Chapter 8 described for the first time competition in hippocampal cells for polarization processes. We found that topography was preferred over chemical ligands in polarization, which suggests that topography exerts a stronger feedback loop in increasing growth from neurites in stage 2 hippocampal cells. It is hypothesized that polarization is dictated by integration of signals at the cell soma, with positive feedback loops at the axonal grown cone and negative feedback loops throughout the neuron to inhibit growth of other neurites [7].

On a different scenario, after axons are formed, controlled steering of axon growth is required to establish connectivity with target cells [8]. This axon guidance has been extensively studied, showing a localized decision at the growth cone in a time period of minutes to 1 hour, a drastic difference from
polarization that occurs in 24-48 h and involves the complete cell body [9]. Integration of multiple signals at the growth cone has been extensively studied with commissural axons that follow a defined trajectory in development of the early spinal cord [10]. In that case, multiple cues including netrins, semaphorins and slits orchestrate axon pathfinding [10,11]. Netrin-1 also effectively attracts developing hippocampal axons [12] and semaphorins induce growth cone collapse in hippocampal cells [13], which supports the effects of external signals in growth cone pathfinding for hippocampal cells. Additionally, multiple in vitro turning assays have focused on how the growth cone detects signals such as gradients of netrin-1 [14], brain derived growth factor (BDNF) [9] and Sema III [15]. Gradients of NGF have also been investigated in the past for directional growth [16-18].

Studies showing an increase in local actin assembly rate [19,20], and local calcium waves that propagate inside the growth cone [9,21] have further supported the integration of signals at the individual growth cone. As this integration is different from polarization processes, it would be interesting to directly compare both phenomena, determining if the already formed axon reacts in a similar way as the polarizing cells to competing extracellular cues. For this analysis, we suggest a competition study where topography and chemical ligands are simultaneously presented to an already formed axon, and the change in axon growth direction would be analyzed.

Although hippocampal cells are not the standard model for axonal pathfinding studies, there is strong evidence showing that axons from these cells can change direction when they encounter specific cues, such as topographical grooves or chemical cues [22]. Because of this, we suggest to create a competition scheme for axon guidance using the same exact techniques used for polarization competition in Chapter 8. Figure 9.2 illustrates a possible procedure
for creating this axon guidance competition geometry. A PDMS substrate would be fabricated containing both microchannels and an additional “pit” for cell micropositioning. This pit would initially consist of a ~20 \( \mu \)m circle connected to an arm ~150 \( \mu \)m long and ~5 \( \mu \)m wide. This specific geometry would theoretically force a hippocampal neuron micropositioned on the circle to form the axon on the arm.

After microfabrication of PDMS substrates containing microchannels and cell pits, PDMS wells would be positioned on PDMS substrates as described in Chapter 8 (Figure 9.2, step 1). In this case, this second PDMS film would be designed to contain multiple wells that would be aligned with the microchannels on the PDMS substrate. The fabrication of the multiple wells might need to be performed with photolithography techniques to better control the size and distribution. Once the PDMS wells are aligned and sealed on PDMS substrates, NGF immobilization could be performed as described in previous chapters (Figure 9.2, step 2). After extensive washing and releasing the PDMS wells (Step 3), a competition geometry for axon guidance would be created, where a cell pit is connected to an area containing microchannels on one side and immobilized NGF on the other side. Micropositioning would be performed to precisely place individual hippocampal neurons inside the pits (i.e., circles) (step 4). Cells would extend axons following the arm and eventually reaching the area containing the stimuli. Axon steering would take place and the change in the direction of growth would be analyzed.
Figure 9.2. Schematic of fabrication process to create competition geometry for analyzing axon guidance. Microchannels and cell pits are fabricated on PDMS using microlithographic techniques. After this, a PDMS film containing multiple wells is aligned along the edge of microchannels under the microscope (1). NGF immobilization using aryl-azido photolinkers (2) is performed inside the PDMS wells. After extensive washing, PDMS wells are removed (3), which finally renders an area modified with chemical ligands parallel to microchannels. Hippocampal neurons are subsequently micropositioned on the cell pits and analyzed for axon steering after reaching the area containing microchannels and immobilized NGF (4).

9.4 References


Appendix A
NGF Signaling Pathways

A.1 TrkA Signaling

After NGF binds to TrkA receptors, these dimerize and autophosphorylate tyrosines that initiate multiple intracellular cascades by binding of adaptors and specific signaling proteins [1]. The most important cascades are summarized in the following sections. Further details can be found in Sofroniew et al. [2].

A.1.1 Ras- MAP Kinase Pathway

Ras is a membrane-targeted GTPase that is activated upon recruitment of specific GTP exchange factors (GEF). TrkA tyrosine phosphorylation activates adaptor proteins that subsequently activate GEFs and ultimately Ras. Activated Ras leads to phosphorylation of kinases of the MAP (mitogen-activated protein) family (Erk 1/2), which finally mediate activation of a transcription factor that produces transcription of the c-fos early gene. This gene is associated with initiation and maintenance of neuronal differentiation (Figure A.1).

A.1.2 PI 3 (phosphotidylinositol-3) Kinase Pathway

PI 3 kinase is activated by phosphorylated tyrosines of TrkA. This molecule further phosphorylates membrane phosphoinositides, PI-3-4-P₂, and PI-2,4,5-P₃, which activate Akt, a serine-threonine kinase that regulates neuronal survival in several ways (Figure A.2).

A.1.3 Phospholipase C-γ (PLC-γ) Pathway

Phosphorylated tyrosines activate PLC-γ, which itself hydrolyzes phosphatidylinositol 4,5-biphosphate (PI 4,5-P₂). This hydrolysis produces two common secondary messengers: IP₃ and diacylglycerol (DAG). The first one mediates release of intracellular calcium, another potent and broad secondary messenger, whereas DAG activates protein kinase C (PKC). These molecules mediate phosphorylation of multiple proteins involved in survival and differentiation.

A.2 p75NTR Signaling

p75NTR modulates in many cases TrkA signaling, but it has independent pathways as well. This receptor predominantly controls cell viability by regulation of apoptosis.

In the ceramide pathways, this lipid secondary messenger is obtained from the hydrolysis of sphingomyelin in the cell membrane. NGF binding to p75NTR activates sphingomyelinase by interactions with a cytoplasmic domain of the receptor. Signaling through ceramide controls neurite formation and neuron polarization, but also produces apoptosis in large concentrations [3].
Figure A.1. Ras-MAP pathway. Ras GTPase is activated by phosphorylated TrkA, which ultimately activates MAP kinases. These produce the activation of transcription factors that promote gene transcription. Figure adapted from [2].

Figure A.2. PI 3 kinase pathway. Phosphorylated TrkA activates PI 3 kinase which produces PIP3. This last molecule activates the kinase Akt, which mediates cascades for survival. Figure adapted from [2].
A.3 NGF Endosome Signaling

NGF-TrkA complex endocytosis has been extensively studied. After NGF binds to TrkA receptors on the cellular membrane, the receptor-ligand complex is internalized and transported to the cell body, which has been shown to play a major role in the intracellular signaling cascades [4]. However, not all signaling is dependent on internalization. Zhang et al. reported dramatic differences in intracellular pathways for internalized receptors versus “surface receptors” [5]. This group demonstrated that non-internalized receptors exhibited phosphorylation of tyrosines after NGF binding that triggered a long-lasting activation of the PI 3- kinase pathway, critical for survival. In contrast, they also demonstrated that endocytosis was essential for differentiation (i.e., MAP kinase pathway). Other studies in compartmentalized neurons also proposed localized mechanisms for neurite outgrowth that do not involve retrograde transport of endosomes [1].

p75NTR receptor also produces clathrin-coated vesicles after NGF binding, but this event occurs at a much slower rate than for TrkA receptors [6]. More importantly, signaling from this receptor does not require endocytosis as sphingomyelin hydrolysis (ceramide pathway) is not reduced when internalization is prevented [7].

A.4 References


Appendix B
Reactivity of Arylazides

NGF immobilization was performed using arylazido (phenylazido) chemistry. This appendix provides information about the high reactivity of nitrenes formed by photolysis of the arylazido functional group with UV light.

### Table B.1. Reactivity of arylazides with functional groups found in proteins

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Aminoacid with Functional Group</th>
<th>Reactivity with Arylazides</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C</td>
<td>All</td>
<td>Do no react</td>
</tr>
<tr>
<td>C-H</td>
<td>All</td>
<td>React (see Figure B.1)</td>
</tr>
<tr>
<td>Aryl</td>
<td>Phe, Tyr, Trp, His</td>
<td>Nitrenes insert into aryl CH</td>
</tr>
<tr>
<td>N-H</td>
<td>Lys, His, N-termini</td>
<td>React (see Figure B.1)</td>
</tr>
<tr>
<td>Guanidinium</td>
<td>Arg</td>
<td>Probably react, but no exp. data</td>
</tr>
<tr>
<td>O-H</td>
<td>Ser, Thr, Tyr</td>
<td>React (see Figure B.1)</td>
</tr>
<tr>
<td>S-H</td>
<td>Cys</td>
<td>React (see Figure B.1)</td>
</tr>
<tr>
<td>S-S</td>
<td>Cys-Cys</td>
<td>Probably react, but no exp. data</td>
</tr>
<tr>
<td>S-CH3</td>
<td>Met</td>
<td>React but unstable</td>
</tr>
<tr>
<td>COOH</td>
<td>Aps, Glu, C-termini</td>
<td>React but unstable</td>
</tr>
<tr>
<td>CONHR</td>
<td>Asn, Gln, peptide bond</td>
<td>No experimental data</td>
</tr>
</tbody>
</table>

Figure B.1. Photochemistry of arylazido group. Arylazides (A) create highly reactive singlet nitrenes (B) when irradiated with UV light. Nitrenes can undergo insertion into many functional groups such as C-H, N-H and other bonds. Figure modified from Scriven EF. Azides and nitrenes, reactivity and utility. Orlando: Academic Press, 1984, p.442.
Appendix C
Photolinker Characterization

Immobilization of NGF was performed using a phenyl-azido derivative. This appendix describes the characterization of the photolinker (H-NMR and FITR), and the characterization of the polyallylamine-azido conjugate (PAA-azido).

C.1 Spectra of N-4-(azidobenzoyloxy)succinimide (photolinker)

Figure C.1. $^1$H-NMR of photolinker in DMSO. Aromatic and succinimide groups correspond to the structure of the photolinker. The area of the succinimide peak is ~ twice the area of the two pairs of hydrogens from the aromatic group, which further corroborates the conjugation reaction. The unlabeled peaks correspond to unidentified secondary products.
C.2 Characterization of polyallylamine-azido (PAA-azido)

The ratio of substitution of phenyl-azido groups per amine group in PAA was determined by measuring UV-Vis absorbance at 270 nm (for determining phenyl-azido groups) and a ninhydrin assay (for determining the total primary amine groups). It was determined that there were \(~35\) phenyl-azido groups per amine group in PAA-azido, which confirms the high degree of reaction.

![FTIR spectrum](image)

**Figure C.2.** FTIR of photolinker in KBr pellet. Stretches for azido group (2133 cm\(^{-1}\)), ester group (1746 cm\(^{-1}\)) and C=O (1630 cm\(^{-1}\)) group (from succinimide) were identified. These correspond to the structure of the photolinker.
Figure C.3. UV-Vis spectrum of PAA-azido in water. Strong absorbance at 270 nm is attributed to the phenyl-azido group conjugated to PAA. Azidobenzoic acid is the initial reactant for producing the photolinker (positive control). Final filtrate corresponds to the final solution that is filtrated during ultrafiltration for purifying PAA-azido.
Appendix D
Experimental Protocols

D.1 Microfabrication of Poly(dimethylsiloxane) (PDMS) Patterns

Background

This protocol describes how to fabricate micropatterns in PDMS. PDMS is a silicone composed of repeating \(-\text{OSi}(\text{CH}_3)_2-\) units that is widely used for soft lithography applications. The basic process in soft lithography consists of several steps. The first procedure is the fabrication of a master using conventional photolithography. For this, a chromium mask with a defined pattern is used for exposing a photoresist (usually SU-8) previously spun down on silicon. Alternatively, the master can be fabricated with Reactive Ion Etching (RIE) after the features are written with Electron beam (E-beam lithography). This master is subsequently used as a mold for performing replica molding by pouring a prepolymer mixture, curing and obtaining the final PDMS piece or “stamp”. The master can be used numerous times for obtaining PDMS structures.

References:

Materials

\textit{E-beam lithography}
- Silicon wafer with 5 \(\mu\text{m}-\text{SiO}_2\) layer WaferWorld
- Poly(methyl methacrylate) (PMMA) resist Microchem 495PMMAC4 (4% in chlorobenzene)
- Methyl-isobutyl-ketone (MIBK) Aldrich 36,051-1
- Isopropyl alcohol (IPA)
- Acetone
- Chrome plated tungsten rods R.D. Mathis CRW-1
- Cr mask etchant (ceric sulfate and nitric acid) Transene

\textit{Silanization of masters}
- tridecafluoro-1,1,2,2-(tetrahydrooctyl) trichlorosilane (MW = 481.55) Gelest BP-84-5117

\textit{Replica Molding}
- Sylgard 184 Kit Dow Corning
Methods

E-beam Lithography

1. SiO₂ layer can be grown on silicon wafers using low pressure chemical vapor deposition (LPCVD) (performed by Dr. Shaochen Chen’s students at Pickle Research Center, UT – Austin, Mechanical Engineering).
2. After growing SiO₂ layer, cut silicon wafer into 1 cm²-pieces using a diamond cutter. Clean pieces by sonication in acetone, isopropyl alcohol and water (10 min each). Blow-Dry with N₂.
3. Spin coating: Pour ~ 200 µL of PMMA resist on wafer and let it spread for 10 seconds. Spin coat for 45 seconds at 3000 rpm (thickness ~400-500 nm).
4. Bake resist for 30 minutes at 170°C in the oven.
5. Scratch a corner of the wafer before writing with the E-beam. This will be used during E-beam for focusing and alignment.
6. Design patterns using the software for the E-beam (2 µm or 1 µm lines, or any other shape)
7. Write structures with a dose of 1 and beam current of 0.2 nA.
8. Develop structures with a mixture of MIBK and IPA (1:3) for 1.5 minutes. Dip wafer in IPA for 1.5 minutes after development. Let it air-dry (Note: if N₂ blow dry is used, be careful not to remove small structures)

Lift-off

1. After development, use the thermal evaporator to evaporate 100 nm of Cr. For this, use a Cr rod.
2. After evaporation, immerse substrate in acetone for 30 min. Check under the reflectance microscope if all the PMMA resist is stripped. If not, sonicate carefully for 1-5 seconds (Note: strong sonication can remove all the patterns form the surface).

Reactive Ion Etching and Silanization

1. Take the substrates with the Cr mask to the etcher. Use a gas mixture of CHF₃ (66%) and O₂ (33%) for etching (150 Watts). The etching rate is ~ 50 nm/min. Monitor thickness with a Nanospec (performed at Pickle Research Center, UT-Austin).
2. After RIE, remove the Cr mask with the Cr etchant for 15 min. Dip substrates in water and blow-dry with N₂.
3. Clean etched wafers by sonicating in acetone and IPA(10 min each).
4. UV-ozone samples for 20 minutes (performed at Dr. Lynn Loo’s lab at UT-Austin, Chemical Engineering).
5. Coat samples with silane by pouring ~ 300 µL of silane compound on the bottom of a Petri dish, and double-tape the substrates to the lid, so the silane evaporates and coats the substrates. Let it evaporate for 10 minutes (Note: silane compound is highly carcinogenic. Use it inside hood only).
6. Clean again substrates in acetone and IPA (10 min each). These substrates are the final masters.
**PDMS Molding**

1. Mix in a plastic cup one part of curing agent per ten parts of base (in weight). Mix thoroughly with a plastic stick. Degas mixture under vacuum to get rid of air bubbles.
2. Pour mixture on a Petri dish with the master in the center of the dish (~10 g /10 cm dish). Let it polymerize for 48 h at room temperature or for 1 h at 70°C.
3. Release carefully PDMS film from substrate.
D.2 Immobilization of NGF

Background

This protocol describes the process for the covalent immobilization of nerve growth factor (NGF) or other protein to a polymer substrate (e.g., poly(dimethylsiloxane), polypyrrole). The chemistry is based on the reactivity of the arylazido group (\(-\mathrm{N}_3\)), which creates a highly reactive intermediate upon exposure to UV light. This group transforms into phenyl nitrene, which can react with neighboring atoms to form a covalent bond. This intermediate can undergo insertion non-specifically into C-H, O-H and N-H bonds. The procedure is based on a method developed by Matsuda et al. (1995) and modified by Ito and colleagues for immobilization of growth factors in particular.


Materials

Preparation of N-4-(azidobenzoyloxy)succinimide (photolinker)
- Dicyclohexylcarbodiimide (DCC) Aldrich D8,002
- Tetrahydrofuran (THF) Fisher T425-4
- N-hydroxysuccinimide (NHS) Aldrich 130672
- 4-azidobenzoic acid (AB acid) TCI America A0930
- Isopropyl alcohol (IPA) Fisher A416-4
- Diisopropyl ether (DIE) Aldrich 185302

Polyallylamine conjugation to N-4-(azidobenzoyloxy)succinimide
- Poly(allylamine hydrochloride) (PAA) Aldrich 283223
- N-4-(azidobenzoyloxy)succinimide (photolinker) previous preparation
- Phosphate buffer saline, PBS (10 mM) Fisher D119-4
- N,N-dimethylformamide (DMF) Centricon plus-20, 10,000 NMWL Millipore UFC2 LGC
- Ultrafiltration filter devices Microcon, 100,000 NMWL Millipore YM-100

Immobilization of NGF
- Nerve growth factor (NGF 2.5S) Promega, G5141
- Polymer substrate (PDMS, polypyrrole)
- HCl 0.01M, pH=2.5
- PBS (10 mM)
- TRIS buffer (10 mM)
Equipments

- Swinging buckets Centrifuge.
- UV lamp (22 mW/cm², 365 nm)

Methods

Preparation of N-4-(azidobenzoyloxy)succinimide (photolinker)
This procedure was modified from Matsuda, Langmuir, 1995, 11: 2272.

1. Dissolve 6.7 g of DCC in 25 mL of THF.
2. Dissolve 3.71 g of NHS and 4.8 g of AB acid in 75 mL THF cooled in an ice bath.
3. Add dropwise DCC solution to NHS + AB acid solution, while stirring.
4. Leave reacting for 3 hours in ice bath.
5. Warm up to room temperature and leave stirring overnight.
6. A white solid is formed. Filter off the solid and wait for the evaporation of the solvent.
7. Take the white solid and dissolve it in a mixture of IPA and DIE (1:1).
8. Allow evaporation of the solvent. A yellowish solid is obtained. This is the photolinker (store in the dark).

Polyallylamine conjugation to N-4-(azidobenzoyloxy)succinimide
This procedure was modified from Ito et al., Bioconjugate Chem. 1998, 9:277-282

1. Dissolve 15 mg of PAA in 10 mL of PBS.
2. Dissolve 13 mg of photolinker in 5 mL of DMF in an ice bath, and add PAA solution.
3. Leave reacting at 4°C for 24 hours.
4. Ultrafiltrate solution with centrifugation filter device (centrifugation at 3500 x g for 10 minutes, 10,000 NMWL). Discard the filtrate.
5. Add 10 mL of DDI water to centrifugation device, ultrafiltrate again and discard filtrate. Repeat this washing three times.
6. Keep the retentate after the fourth ultrafiltration. The typical final volume is about 200-300 µL of clear solution (PAA-azido solution).
7. Ultrafiltrate the retentate one more time with a 100,000 NMWL microcon filter (14,000 X g for 12 minutes in microcentrifuge). Keep the filtrate (200-300 µL).
8. Dissolve final filtrate in DDI water. For immobilization on polypyrrole, do not dissolve. For immobilization on PDMS dissolve in ~2 mL (1:8).
9. If desired for analysis, measure UV-VIS absorbance of the final filtrate to check the presence of the azido compound at 280 nm.

Immobilization of NGF

1. Clean the substrate (PDMS or polypyrrole) and place it in a clean Petri dish.
2. Typically, add 50 µL of the PAA-azido solution to the substrate surface and leave drying overnight.
3. Expose the first cast layer of PAA-azido to UV light for 15 seconds.
4. Wash with HCl pH = 2.5 tree times to remove unreacted compounds.
5. Wash with PBS two times.
6. Add a second layer of PAA-azido solution (50 µL). Leave drying overnight or for ~ 12 hours.
7. Typically, dissolve 20 µL of NGF-2.5s in 30 µL of TRIS buffer (depending on the desired concentration). Cast this solution by adding the 50 µL on top of the substrate. Leave drying for ~ 12 hours.
8. Expose to UV light or UV laser. If a mask (e.g., TEM grid) is used, place the mask on top of the substrate before exposing. Typically expose for 15 seconds.
9. Wash with PBS six times, leaving each time the solution for 5 minutes before aspirating.
10. Wash twice with DDI water.
11. Air dry samples.
12. Analyze immobilization (if NGF was fluorescently labeled with FITC, use the fluorescence microscope to check the presence of the protein. If not, use immunochemistry methods (see additional protocol).

Notes
- The synthesis of the photolinker usually produces enough product for numerous reactions with PAA.
- Phenyl-azido group absorbs strongly at 280 nm. It is possible to check the presence of the azido group by UV-VIS spectroscopy.
- The literature protocol for the conjugation of PAA to photolinker used the opposite ratio of solvents (DMF: PBS, 2:1). However, the proposed ratio in this protocol gives better results in the ultrafiltration process, which was usually much more difficult to filtrate properly when more DMF was added. In addition, protocols for other commercial available photolinkers (e.g., Pierce, 21451) use lower levels of organic solvent as well (~10%).
- Preferentially, do all the procedure in the dark or protected from light.
- For samples for cell culture, do all the immobilization steps steriley inside horizontal flow hood and with sterile solutions.
D.3 Labeling of NGF with FITC

Background

Fluorescein is a fluorophore that absorbs at 494 (blue) nm and emits at 518 nm (green). It is commonly used as a fluorescent tag for proteins. Fluorescein isothiocyanate (FITC) is a reactive form of the fluorophore, which has an isothiocyanate functional group that reacts with amine groups. By incubating FITC with proteins it is possible to attach fluorescein to the protein as a fluorescent label, and use this for detection.

References: Molecular Probes Product Information for "Amine-Reactive Probes"

Materials

- NGF 2.5S (100 µg/ml) Promega G5141
- Fluorescein isothiocyanate (FITC) Molecular Probes F1906
- Dimethyl sulfoxide (DMSO) Sigma
- Sodium bicarbonate Fisher
- Size exclusion chromatography Biorad, 732-6221 columns (exclusion limit 6,000 Da)

Methods

1. Add 40 µL of sodium bicarbonate buffer (0.1 M, pH = 9) to 100 µL of NGF in a 2 ml tube.
2. Add 1.2 mg of FITC to 100 µL of DMSO.
3. Take 10 µL of FITC solution and add it to NGF in bicarbonate buffer. Wrap up tube in aluminum foil.
4. Put tube in orbital shaker for ~ 10 h at 4°C.
5. Separate conjugate from unreacted FITC using size exclusion chromatography columns (75 µL/column). Invert columns to resuspend gel and remove the excess of buffer. Add 75 µL of reaction mixture and centrifuge for 4 minutes at 1000 x g.
6. After recovering NGF from the columns, measure conjugation efficiency and degree of labeling with UV-Vis by measuring absorbance at 280 nm and 494 nm.
7. Calculate protein concentration and degree of labeling

\[ A_{protein} = A_{280} - \frac{A_{494}}{3} \]

Concentration protein (mg/mL) = \[ \frac{A_{Protein}}{\varepsilon} = \frac{A_{Protein}}{1.42} \]

Concentration protein (M) = concentration (mg/mL) / 26,000

Concentration FITC (M) = \[ \frac{A_{494}}{\varepsilon} = \frac{A_{494}}{68000} \]

Degree of labeling = moles dye/moles protein = Concentration dye (M)/ concentration protein (M).
D.4 NGF ELISA

Background

ELISA (enzyme-linked immunosorbent assay) is used to quantitatively detect an antigen by using specific antibodies that recognize it. A sandwich ELISA consists of first immobilizing a primary antibody to an ELISA plate, followed by incubation of the antigen, incubation with of a second primary antibody and incubation with a secondary antibody conjugated to an enzyme. By adding a specific substrate for the enzyme, a colored product is produced, which is detected by absorbance. ELISA sensitivity is ~10 pg/mL of antigen.

References: NGF Emax product information, Promega Technical Bulletin # 226

Materials

- 96 well ELISA plates Corning Incorporated 3590
- NGF E_{max} ImmunoAssay System Kit Promega G7630
- HCl, 1N
- Carbonate coating buffer
  (25 mM sodium bicarbonate, 25 mM sodium carbonate)
- Tris washing buffer (TBST wash buffer)
  (20mM Tris-HCl,150mM NaCl, 0.05% Tween®20)

Equipments

- Plate shaker.
- Plate reader with 450 nm filter.
- Multichannel pipette.

Methods

(Modified from Promega Technical Bulletin # 226)

1. Mix 2 µL of anti-NGF primary antibody (Anti-NGF pAB) per 12.5 mL of carbonate buffer. Mix thoroughly and add 100 µL per well.
2. Seal wells with parafilm and a plate lid. Incubate overnight at 4°C.
3. Prepare Block & Sample buffer (B&S) 1X (this comes with the kit), by diluting 5X stock in DDI water.
4. Flick out contents of the plate in the sink, and slap the plate upside down three times on a paper towel.
5. Wash wells with TBST buffer with 200 µL per well, flick out contents, and slap plate upside down.
6. Add 200 µL of B&S buffer to each well. Incubate at room temperature for 1 h.
7. Prepare samples and standards for calibration curve. NGF standard comes with the kit with a concentration of 1 µg/mL. Dilute the supplied NGF 1:2000 in B&S buffer (500 pg/mL). Dilute accordingly to create the desired solutions (in duplicate) for the calibration curve (Recommended range: 7.8-500 pg/mL). Also,
dilute 1:4 samples in B&S buffer. (Note: Previous sample dilution might be necessary for obtaining final concentrations within the standard calibration range. Estimate NGF concentrations to identify dilution factors).

8. Flick out contents of the plate in the sink, and slap the plate upside down three times on a paper towel.

9. Wash wells with TBST buffer with 200 µL per well, flick out contents and slap plate upside down.

10. Add 100 µL of NGF samples and standards (in duplicate) to plate. Preferentially, add samples in duplicate and with different dilution factors if required.

11. Seal wells with parafilm and a plate lid. Incubate for 6 h at room temperature on plate shaker.

12. Wash all wells with TBST buffer five times.

13. Mix 2.5 µL of anti-NGF antibody (Anti-NGF mAb) per 10 mL of B&S buffer. Mix thoroughly and add 100 µL per well.

14. Seal wells with parafilm and a plate lid. Incubate overnight at 4°C.

15. Wash all wells with TBST buffer five times.

16. Prepare fresh 1X B&S buffer by diluting 5X stock in DDI water.

17. Mix 100 µL of stock anti-rat IgG, HRP conjugate per 9.9 mL of B&S buffer. Mix thoroughly and add 100 µL per well.

18. Seal wells with parafilm and a plate lid. Incubate for 2.5 h at room temperature on plate shaker.

19. Wash all wells with TBST buffer five times.

20. Equilibrate TMB (enzyme substrate) solution to room temperature. Add 100 µL per well and incubate for 10 minutes at room temperature on plate shaker.

21. Stop reaction by adding 100 µL of HCl 1N. (Note: blue color changes to yellow).

22. Record absorbance of plates with a plate reader at 450 nm. (Note: read absorbance within 30 minutes of adding HCl).

23. Plot calibration curve (Absorbance vs. concentration). Determine NGF concentration from samples by using calibration curve (take into account the dilution factors). Only analyze samples with absorbance values within the range of the calibration curve.

Notes
- The Promega kit contains Anti-NGF ab, Block and Sample buffer (5X), NGF standard, Anti-NGF mAb, Anti-Rat IgG, HRP conjugate and TMB solution.
- Never touch or scratch the bottom or sides of the wells. Be very careful with the pipette tips while adding solutions.
- Prepare enough TBST buffer for all wells and washes.
- Mix antibodies and solutions according to the number of wells analyzed. The kit has reagents for 2 full 96-well plates, but these can be used in multiple runs if fewer samples are analyzed. Maintain kit contents at 4°C all the time and use these for three months at the most.
D.5 Hippocampal Neuron Culture

Background

Embryonic hippocampal neurons (E18) are the most common model for polarization (i.e., axon formation) mechanisms. These cells are pyramidal neurons that are generated during embryogenesis in the ventricular surface and are migrating to the pyramidal cell layer. These cells are post-mitotic but are not extending axons yet. Hippocampal neurons polarize in culture by determining a single axon and several dendrites. This event spontaneously occurs in culture during the first 48-72 h, during which neurons follow well-defined stages, from stage 1 where cells are unpolarized, to stage 3 where cells establish an axon.

References:  Banker GA, Cowan M. Brain Res. 1977; 126: 397-425.  
Brainbits trituration protocol.

Materials

- Hippocampus tissue  
- Papain  
- Neurobasal Medium  
- Hibernate E Medium  
- B-27 supplement  
- L-glutamine  
- L-glutamic acid  
- Antibiotic-antimyotic  
- Trypan blue  
- Fire-polished Pasteur pipettes

Brainbits  
Worthington LS003119  
Invitrogen 21103  
Brainbits  
Invitrogen 17504  
Fisher BP379-100  
Sigma G 1251  
Sigma A7292  
Sigma T8154

Methods

Cell Culture Medium Preparation  
(recipe for 25 mL)

1. Dissolve 0.091 g of L-glutamine in 5 mL of DDI water. Mix thoroughly. Filter-sterilize.
2. Dissolve 0.046 g of L-glutamic acid in 5 mL of HCl 1M. Mix thoroughly. Filter-sterilize.
3. Mix 25 mL of Neurobasal medium, 500 µL of B-27, 100 µL of L-glutamine solution, 10 µL of L-glutamic acid solution and 250 µL of antibiotic-antimyotic solution.
Cell Trituration and Culture  
(adapted from protocol provided by Brainbits)

1. Mix 1 mL of Hibernate E medium with 20 µL of B-27 in a centrifuge tube.  
2. In a separate tube, dissolve 4 mg of papain in 1 mL of Hibernate E medium.  
3. Take out from the refrigerator the hippocampus tissue (The tissue is stored in 2 mL of Hibernate E medium with B-27)  
4. Remove 1 mL of medium from the tube containing the tissue and keep in a separate centrifuge tube.  
5. Add 1 mL of Hibernate medium with papain to the tube with the tissue.  
6. Incubate for 20 minutes in a water bath at 30°C.  
7. Remove 1 mL of medium from the tube.  
8. Add 1 mL of Hibernate E with B-27. Mix gently by inverting tube.  
9. Remove 1 mL of medium from tube with tissue.  
10. Using a fire-polished Pasteur pipetter, triturate the tissue by aspirating and releasing the remainder 1 mL of media with tissue. Repeat this process at least 10 times or until tissue is totally dispersed.  
11. Add back the 1 mL of media that was removed in step 4.  
12. Wait 1 minute for settlement of undispersed big pieces of tissue.  
13. Transfer the 2 mL with cells to a 15 mL centrifuge tube. Try not to aspirate the big tissue fragments, if any.  
14. Centrifuge 1 minute at 200 x g.  
15. Remove supernatant carefully, leaving ~ 500 µL of media with cell pellet.  
16. Add 1 mL of prepared Neurobasal medium. Mix 1-3 times with pipette to distribute cells.  
17. Aspirate 20 µL of the cell suspension and mix with 20 µL of trypan blue.  
18. Count cells in the hemocytometer. Blue cells are dead cells. Typical total yield is between 500,000 -1,000,000 cells.  
19. Add more Neurobasal medium preparation according to desired cell culture density and mix gently. Typically, dissolve for obtaining 300-500 cells/µL and add ~30,000-50,000 cells per 3-cm dish.  
20. Put dishes in incubator at 37°C and 5% CO₂. Typically, fix and analyze cells after 24-48 h, depending on each particular experiment.

Notes
- Prepare medium the same day of cell trituration or the day before.  
- For fire-polishing Pasteur pipettes, use an alcohol lamp or regular lighter inside the horizontal hood, put the tip of the Pasteur pipette next to the flame, and wait 5-10 seconds while continuously turning the tip. Be careful not to seal it completely.  
- Use tissue within 5 days of received.  
- In steps 3-9 be careful not to remove the tissue when aspirating with the pipette (do not touch the bottom of the tube).
D.6 PC12 Cell Culture

Background

PC12 cells are derived from a transplantable rat pheochromocytoma. These cells extend neurites in the presence of NGF and grow in clusters when are not differentiated. It is recommended to use these cells between passages 15 and 50. They adhere poorly to plastic, and therefore it is recommended to culture them in collagen-coated dishes.


Materials

- Collagen I (1 mg/mL) Sigma C7661
- Ethanol 30%  
- F12K medium (Kaighn’s modification of Ham’s 12 medium with 2 mM L-glutamine) Sigma N3520
- Sodium bicarbonate
- Horse Serum (Heat inactivated) (HS) HyClone
- Fetal Bovine serum (FBS) HyClone
- Penicillin-streptomycin (Pen/strep)
- Trypsin-EDTA (TRED)
- NGF 2.5S (100 µg/ml) Promega G5141

Methods

Collagen Coating of Dishes

1. Dilute 27.5 µL of collagen in 2 mL of 30% ethanol for each 10 cm dish.
2. Add 2 mL of collagen/ethanol solution to each 10 cm dish.
3. Leave dishes cracked in TC hood overnight to dry.
4. Store at -20°C.

Cell Culture Medium Preparation

Stock solution
1. Reconstitute F12K medium in 1 L of water. Leave stirring for 1 hour.
2. Add 1.5 g/L of sodium bicarbonate.
3. Adjust pH to 7.1-7.4.
4. Filter sterilize and store at 4 °C.

Serum containing medium (15% HS, 2.5% FBS)
1. For 200 mL of medium: Mix 165 mL of stock solution, 30 mL HS, 5 mL FBS and 2 mL Pen/strep.
Culture

1. Passage them when 70% confluent. Use TRED to detach cells from dish. Triturate several times before dispensing cells in new dishes. Use 1:8 split ratio.
2. Feed cells every 2-3 days.
3. When cells are extremely clustered, it is possible to use a needle and syringe in the trituration step to brake up clumps and disperse individual cells. This is recommended specially when cells are cultured on experimental samples.
4. For differentiation and neurite extension, add 50 ng/mL of NGF to medium immediately before feeding cells (Do not store medium with NGF). Cells will begin extending small neurites after ~3 days of adding NGF. Evident neurite extension is observable after ~1 week with NGF.

Notes
- Doubling time: 92 h.
- Some cells also adhere to TC dishes, but it is recommended to use collagen-coated dishes.
- Depending of the experiment, cells can be primed with NGF (50 ng/mL) for 1 week before plating these on the experimental samples. This procedure facilitates neurite extension in 1-2 days after plating.
D.7 Cell Micropositioning

Background

Micropositioning techniques are used to place individual cells in specific locations. This procedure is commonly used for creating artificial neural networks. The setup consists of a glass capillary connected to a microinjector and mounted on a micropositioner. These are mounted on a reflectance microscope located inside a horizontal hood. The capillary is used to aspirate single cells and precisely releasing these on a desired location.


Materials

- Hippocampal cell suspension               Protocol for Hippocampal Neuron Culture
- Neurobasal medium with B-27, L-glutamine, L-glutamic acid and antibiotic-antimycotic Protocol for Hippocampal Neuron Culture
- Single-barrel standard borosilicate glass tubing (OD = 1 mm, ID= 0.58 mm) World Precision Instruments 1B100-4

Equipments

- Fixed-stage upright microscope/ reflected system (Olympus BX51WI)
- Joystick Micromanipulator (Narishigue, MN-151)
- Pneumatic injector (Narishigue, IM-9C)
- Pipette puller (Narishigue, PP-830)

Methods

Pulling of Capillaries

1. Turn the power switch on (heater level display shows a value of 25).
2. Open the semicircular acrylic hood and loosen the metal knobs. Insert a clean glass capillary and tight it with the knobs.
3. Move up the bottom part of the puller with the weights attached to it, loosen the knobs, and tight the bottom part of the pipette.
4. Press the start switch to begin the pulling (heater level increases from 25 to 55).
5. Take out the two pulled capillaries by loosen the knobs.

Cell Micropositioning

1. Connect a pulled capillary to the microinjector tubing, and mount it on the micropositioner.
2. Place substrate where cells are going to be positioned (e.g., PDMS film) in a 10-cm Petri dish (not cell culture dish).
3. Add cell suspension in culture medium (3-5 x 10^5 cells/mL) only on the periphery of the substrate.
4. Allow cells to settle for ~10-15 minutes.
5. Move the dish inside the hood with microscope and micropositioner.
6. Before performing the micropositioning, add more medium to the Petri dish to cover the substrate surface.
7. Identify a single neuron on the surface of the Petri dish (outside the substrate). Aspirate it with the micropipette by creating suction with the injector.
8. Move the capillary with the micromanipulator, and position it on the substrate area where the cell is going to be released.
9. Release the cell from the capillary. Adjust the final location of the cell by sucking and releasing medium next to the cell.
10. Wait ~2 minutes for the cell to adhere to the substrate before moving the capillary again.
11. Repeat the same procedure for ~6-8 cells in a maximum of 30 minutes.
12. Move the dish very carefully to the incubator.
13. Continue regular cell culture procedure.

Notes
- Do not place the substrate in the center of the 10-cm dish, but slightly toward the edge. This allows to have more room for the micropositioning.
- Do micropositioning for a maximum of 30 minutes to minimize the time cells are out of the incubator.
- Cells do not completely attach to the substrate after micropositioning. Be careful when moving the pipette back and forth so cells do not move away from the desired location.
- When moving cells back to the incubator, be extremely gentle with the movement as cells are not completely attached and could move away from the desired location.
- For hippocampal neurons, 4-6 substrates can be consecutively used for micropositioning. This is ~2-3 h after cell trituration. For longer times, it is difficult to aspirate cells because these are adhered to the dish surface. Keep dishes inside the incubator (without adding medium on top of the substrate) while doing micropositioning on other dishes.
D.8 Immunostaining of Tau-1, NGF and Laminin in Hippocampal Cells

Background

Embryonic hippocampal (E18) neurons are commonly used in experiments for analyzing polarization (i.e., formation of an axon). One of the most classical methods to identify axons is by immunostaining of Tau-1 (i.e., dephospho-tau). Tau is a microtubule-associated protein that is primarily compartmentalized in axons. In culture, Tau is also present in the cell body and dendrites, but it is highly dephosphorylated in axons, which is translated in a brighter staining, usually with a spatial gradient from the cell body to the growth cone. In addition to Tau-1, this protocol also describes the immunostaining of NGF and laminin (proteins used to influence polarization mechanisms).


Materials

- Paraformaldehyde Sigma P-6148
- Sucrose
- Triton X-100 Sigma X100
- Bovine serum albumin (BSA) Jackson ImmunoResearch
- Mouse anti-Tau-1 antibody Chemicon MAB3420
- Rabbit anti-NGF antibody Abcam ab9208
- Rabbit anti-Laminin antibody Sigma L 9393
- Goat anti-rabbit, TRITC conjugated antibody Sigma T 6778
- Goat anti-mouse, Alexa 488 conjugated antibody Molecular Probes A-11001
- PBS 1X (10 mM) and 2X (20 mM).
- NaOH 1M.

Methods

1. Fixative preparation (for 1.5 mL): Mix 0.75 mL of 2X PBS, 0.75 mL of 8% sucrose (0.08 g/mL DDI water), 0.06 g of paraformaldehyde, and 10 µL of NaOH 1 M in a closed vial. Place vial in water bath at 60°C until the solution becomes clear.
2. Wash live cells with warmed 1X PBS (37°C).
3. Add fixative to cells (~ 100 µL/ 1 cm²). Incubate 20 minutes at room temperature.
4. Wash fixed cells with 1X PBS.
5. Add 2% BSA , 0.1% Triton-X100 in PBS to samples and incubate 20 minutes at room temperature (cell membrane permeabilization).
6. Wash cells with 1X PBS.
7. Add 2% BSA in PBS. Incubate 1h at 37°C.
8. Remove 2% BSA in PBS.
10. Incubate overnight at 4°C.
11. Wash cells with 1X PBS (two times, 5 minutes each).
12. Centrifuge secondary antibodies in microcentrifuge at 4°C for 20 minutes (8,000 x g). For this, add first ~200 µL of 2% BSA in PBS to each 5-10 µL antibody aliquot.

13. Aspirate antibody solution supernatants and dilute in 2% BSA in PBS.

14. Add secondary antibodies diluted in 2% BSA in PBS (1:200) to cells. Usually, prepare one solution with Alexa 488 and TRITC conjugated antibodies (Note: this solution is used for Tau-1 staining combined with either NGF or laminin staining).

15. Incubate for 1 h at 37°C.

16. Wash cells with 1X PBS (two times, 5 minutes each).

17. Add 1X PBS to samples and image.

Notes

- When preparing the fixative, temperature of water bath should not be higher than 60°C.
- Use fixative within 12 h of preparation (it degrades over time).
- Steps 12 and 13 are not required, but centrifugation of secondary antibodies helps removing large aggregates of fluorescent antibodies.
- After fixation, cells can be stored at 4°C for weeks. However, after immunostaining, fluorescence intensity might decrease in a couple of days.
D.9 Actin Labeling with Phalloidin

Background

Phalloidin is a fungal toxin that specifically binds to actin filaments. Fluorescent conjugates of phalloidin are commonly used for visualization of actin in cells.


Materials

- Paraformaldehyde Sigma P-6148
- Sucrose Sigma
- Triton X-100 Sigma X100
- Bovine serum albumin (BSA) Jackson ImmunoResearch
- Phalloidin-TRITC (0.1 mg) Sigma P1951
- PBS 1X (10 mM) and 2X (20 mM).
- NaOH 1M.

Methods

1. Fixative preparation (for 1.5 mL): Mix 0.75 mL of 2X PBS, 0.75 mL of 8% sucrose (0.08 g/mL DDI water), 0.06 g of paraformaldehyde, and 10 µL of NaOH 1 M in a closed vial. Place vial in water bath at 60°C until the solution becomes clear.
2. Wash live cells with warmed 1X PBS (37°C).
3. Add fixative to cells (~ 100 µL/ 1 cm²). Incubate 15 minutes at room temperature.
4. Wash fixed cells with 1X PBS.
5. Add 2% BSA , 0.1% Triton-X100 in PBS to samples and incubate 5 minutes at room temperature (cell membrane permeabilization),
6. Wash cells with 1X PBS.
7. Add 2% BSA in PBS. Incubate 30 minutes at room temperature.
8. Remove 2% BSA in PBS.
9. Dissolve a 5 µL aliquot of phalloidin-TRITC (see Note below) per 1 mL of PBS.
10. Add phalloidin solution to samples (~100 µL/ 1 cm²).
11. Incubate for 30 minutes at room temperature.
12. Wash cells with 1X PBS (two times, 5 minutes each).
13. Add 1X PBS to samples and image.

Notes

- Phalloidin is highly toxic. Use gloves.
- Phalloidin-TRITC aliquots: Dissolve 0.1 mg of phalloidin-TRITC in 200 µL of methanol. Divide into 5 µL aliquots and store at -20°C.
- For preparing fixative, temperature of water bath should not be higher than 60°C.
- Use fixative within 12 h of preparation (it degrades over time).
- After fixation, cells can be stored at 4°C for weeks. However, after staining, fluorescence intensity might decrease in a couple of days.
D.10 Preparation of Cells for Scanning Electron Microscopy (SEM)

Background

Scanning electrode microscopy (SEM) is often used for high-magnification imaging of cells. As SEM is under vacuum, previous dehydration of cells is required, followed by coating with a metal film for making the samples conductive.


Materials

- Paraformaldehyde Sigma P-6148
- Sucrose Sigma
- PBS 1X (10 mM) and 2X (20 mM)
- NaOH 1M.
- Ethanol
- Hexamethyl-disilazane (HMDS) Sigma H-4875

Equipments

- Metal sputter.

Methods

1. Fixative preparation (for 1.5 mL): Mix 0.75 mL of 2X PBS, 0.75 mL of 8% sucrose (0.08 g/mL DDI water), 0.06 g of paraformaldehyde, and 10 µL of NaOH 1 M in a closed vial. Place vial in water bath at 60°C until the solution becomes clear.
2. Wash live cells with warmed 1X PBS (37°C).
3. Add fixative to cells (~ 100 µL/ 1 cm²). Incubate 20 minutes at room temperature.
4. Wash fixed cells with 1X PBS.
5. Prepare ethanol solutions in DDI water with the following concentrations: 30%, 50%, 70%, 85%, 90%, 95% and 100%.
6. Follow these incubation times:
   
<table>
<thead>
<tr>
<th>Ethanol Concentration</th>
<th>Incubation Time (minutes)</th>
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<tr>
<td>30</td>
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7. Add 10 µL of HMDS per 1 cm² of substrate.
8. Air-dry samples.
9. Use sputter to deposit a thin metal film on the samples (e.g., Cr, Au, Pd-Au).
Notes

- When preparing the fixative, temperature of water bath should not be higher than 60°C.
- Use fixative within 12 h of preparation (it degrades over time).
- After fixation and dehydration, cells can be stored at room temperature, preferentially in a dissecator.
- When dehydrating cells on poly(dimethyl siloxane) substrates, be careful when adding HMDS, as this polymer significantly shrinks and bends for larger volumes of this chemical.
D.11 Polypyrrole Polymerization

Background

PPy is commonly electrochemically polymerized, producing thin films on conductive electrodes. The electropolymerization setup usually consists of a standard three electrode configuration (working, counter and reference electrodes) in an electrochemical bath of monomer and an electrolyte in an appropriate solvent. Monomer units adsorb at the positively charged working electrode and oxidize to form pyrrole cationic radicals that subsequently polymerize.


Materials

- Gold wires
- Tungsten boats
- Chrome plated tungsten rods
- Glass slides
- Pyrrole  Sigma 131709
- Poly(sodium-4-styrene sulfonate) (PSS) (70 kDa)  Aldrich 243051
- Alumina
- Glass wool
- Platinum mesh

Equipments

- Thermal evaporator (Denton)
- Bi-Potentiostat
- Saturated calomel electrode
- Current integrator
- Multimeter

Methods

Gold Coating of Slides

1. Take clean glass slides, one chromium rod and gold wire to the thermal evaporator.
2. Make a pellet with the gold wire and place in a tungsten boat. Place the boat and the Cr rod in two different compartments of the thermal evaporator.
4. Evaporate 3 nm of Cr.
5. Evaporate 30 nm of Au.
**Polymerization**

1. Take glass wool and pack it tightly in a Pasteur pipette (half the volume of the pipette). Then add alumina to fill the other half of the column.
2. Add 2.5 mL of pyrrole to the Pasteur pipette and collect the purified pyrrole (> 1.4 mL) in a different container.
3. Mix 200 mL of DDI water with 4 g of PSS and 1.4 mL of purified pyrrole. Stir vigorously.
4. Flame platinum mesh using a Bunsen burner to remove impurities.
5. Prepare a three-electrode set-up for polymerization, as illustrated in Figure D.1. Connect working electrode to gold-coated slide, counter electrode to platinum gauze and reference electrode to saturated calomel electrode. Gold-coated should face the platinum gauze.
6. Perform polymerization in galvanostat with 720 mV of offset voltage, 10 mA/V and 1000 seconds for time constant.
7. For beginning polymerization, switch from “DUMMY” to “NORMAL” in the potentiostat and from “RESET” to “INTEGRATE” in the current integrator, simultaneously.
8. Wait until the multimeter reads the desired voltage depending on the required film thickness. For 200 nm thickness, polymerize up to 85 mV.
9. To stop polymerization, switch back to “DUMMY” and “RESET”.

![Figure D.1. Three-electrode setup for PPy electropolymerization.](image)

**Notes**

- Platinum mesh should be as parallel as possible to goal-coated slide and reference electrode. All components should not touch the bottom or walls of the beaker. Alligator clips should not touch the solution.
D.12 Polypyrrole Patterning

Background

Pyrrole monomer only polymerizes on substrates that are electrically conductive. Based on this principle, the fabrication of PPy microchannels was performed by first patterning an insulting resist on a conductive substrate using e-beam lithography and then polymerizing on the exposed conductive areas.


Materials

- Indium tin oxide glass slides (ITO slides) Delta Technologies
- 1% poly(methyl methacrylate) Microchem 495PMMA-A
- PMMA resist in anisole
- Pyrrole Sigma131709
- Poly(sodium-4-styrene sulfonate) (PSS) (70 kDa) Aldrich 243051
- Alumina
- Glass wool
- Platinum mesh

Equipments

- Spin coater
- E-beam
- Bi-Potentiostat
- Saturated calomel electrode
- Current integrator
- Multimeter

Methods

E-beam Lithography

1. Cut ITO slides in 2 x 2 cm pieces with a diamond cutter.
2. Spin coating: Pour ~400 µL of PMMA resist on ITO slide and let it spread for 10 seconds. Spin coat for 45 seconds at 3000 rpm (thickness ~ 10 nm).
3. Bake resist for 1 h at 160°C in the oven.
4. Scratch a corner of the slide before writing with the E-beam. This will be used during E-beam for focusing and alignment.
5. Design patterns using the software for the E-beam (2 µm or 1 µm lines, or any other shape)
6. Write structures with a dose of 0.6 and beam current of 0.1 nA.
7. Develop structures with a mixture of MIBK and IPA (1:3) for 1 minute. Dip wafer in IPA for 1.5 minutes after development. Let it air-dry (Note: if N₂ blow dry is used, be careful not to remove small structures)
Polymerization

1. Take glass wool and pack it tightly in a Pasteur pipette (half the volume of the pipette). Then add alumina to fill the other half of the column.
2. Add 300-2500 µL of pyrrole to the Pasteur pipette and collect the purified pyrrole (175 -1400 µL) in a different container (depending on the desired concentration).
3. Mix 200 mL of DDI water with 0.5-4 g of PSS and 175-1400 µL of purified pyrrole (depending on the desired concentration). Stir vigorously.
4. Flame platinum mesh using a Bunsen burner to remove impurities.
5. Clean the top part of the PMMA-coated slide with acetone (not the area with patterns). Attach the alligator clip to this clean area.
6. Prepare a three-electrode set-up for polymerization, as described in PPy polymerization protocol.
7. Perform polymerization in galvanostat with 720 mV of offset voltage, 50µA/V - 1 mA/V (depending on the desired current) and 1000 seconds for time constant.
8. For beginning polymerization, switch from “DUMMY” to “NORMAL” in the bi-potentiostat and from “RESET” to “INTEGRATE” in the current integrator, simultaneously.
9. Wait 10 or 30 seconds.
10. To stop polymerization, switch back to “DUMMY” and “RESET”.

Notes

- For spin coating, make sure to add the resist to the ITO side of the slide.
- Face side with patterns of the PMMA-coated slide to the platinum mesh.
Glossary

Actin filaments: Protein filament formed by the polymerization of globular actin molecules. A major constituent of the cell cytoskeleton.

Apoptosis: Programmed cell death.

Axon: Long nerve cell extension that is capable of conducting nerve impulses.

Arylzido group: -N₃, photosensitive group that reacts non-specifically upon UV exposure to create covalent bonds.

Biomimetic: Characteristic that imitates the natural conditions that exist in the body.

Chemotaxis: Directed movement of a cell or organism towards a gradient of a diffusible chemical.

cAMP: Cyclic adenosine 5’-monophosphate. Secondary messenger in intracellular signaling.

Ceramide: Lipid secondary messenger in cells.

Contact (physical) guidance: Cell alignment and growth in response to topography in the substrate.

Dendrite: Small and branched extensions in neurons that receive the electrical signals in synapses.

Developmental Embryology: Science that investigates the succession of changes that take place as a fertilized egg gives rise to an adult or mature organism.

E-beam lithography: Patterning technique in which a beam of electrons is used to write specific structures in a polymeric resist, by producing chain scission of the backbone.

ELISA: Enzyme-linked immunosorbent assay that uses antibodies to detect antigens.

Endocytosis: Internalization of ligand-receptor complexes into vesicles in the cytoplasm.

Etching: Removal of a material with solvents (wet etching) or chemical and physical processes (dry etching).

Extracellular Matrix (ECM): Family of proteins that surround cells in tissue.

Filopodia: Actin-based structure that look like finger-like protrusions.

FITC: Fluorescein isothiocyanate. A reactive form of the green fluorophore fluorescein.
Focal adhesion: Anchoring cell junction to the extracellular matrix mediated by integrins linked to actin filaments.

Growth Cone: Actin-rich highly motile structure located at the tip of an axon.
Growth factor: Extracellular polypeptide signal molecule than can simulate a cell to grow or proliferate.

GTPases: Monomeric GTP-binding proteins.

Lamellopodia: Actin-based structure that look like a veil.

Laminin: Extracellular matrix protein found in basal lamina. It promotes neurite extension.

Lift-off: Patterning technique in which a metal thin film is evaporated onto an pre-exposed and developed photoresist, followed by dissolving the resist and forming metal patterns on the substrate.

Microtubules: Long cylindrical filaments formed by the protein tubulin. They are part of the cell cytoskeleton.

Nerve growth factor (NGF): Growth factor of the neurothophin family that promotes neurite outgrowth and orientation.

Nerve regeneration: Process that take place after an axon in the Peripheral nervous system is severed.

Neural Network: Array of neurons on artificial electrodes that create synapses and communicate.

Neurite (process): Extension of a neuron, including both axon and dendrites.

Neurite outgrowth: Growth of an axon or dendrite.

Neuron: Nerve cells.

PC12 cells: Rat pheochromocytoma cells.

Photolithography: Patterning technique that uses a mask and photosensitive polymers (usually to UV light) to write structures on a substrate.

Polarization: Formation of one axon and multiple dendrites in neurons.

Polyallylamine: Cationic polyelectrolyte composed of – [CH₂- CH(CH₂NH₂)]- units

Poly(dimethylsiloxane) (PDMS): Silicone elastomer composed of –[OSi(CH₃)₂]- units.

Polypyrrole: Electrically-conducting polymer.
PMMA resist: Poly(methyl methacrylate) resist that is sensitive to e-beam irradiation.

Replica molding: Process by which a material is poured and cured into a mold with a specific shape, acquiring the negative-replica of the pattern imprinted on the mold.

Retrograde transport: Transport from the neurites to the cell body.

RIE (Reactive Ion etching): Dry Etching technique composed of a combination of high energy ion bombardment and chemical reaction.

Soft lithography: Patterning technique that uses elastomers as the substrate material.

Soma: cell body.

Schwann cell: Glial cell in the Peripheral Nervous System that wraps around axons to provide electrical insulation.

Stage-3 neuron: Cell that formed a single axon.

SU-8: Negative photoresist that crosslinks.

Synapsis: Communication between neurons that transmits electrical signals from the end of an axon to dendrites in the other cell.

Tissue Engineering: Field that combines principles from engineering and biology to develop implantable scaffolds and devices.

Tau-1: Microtubule associated protein that is primary compartmentalized in axons.

TRITC: Tetramethylrhodamine B isothiocyanate. A reactive form of the red fluorophore rhodamine.
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VITA

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From 1997-2002, she attended Universidad de Los Andes, Bogota, Colombia, obtaining a B.S. in Chemical Engineering with honors. She began her graduate studies in 2002 at the University of Texas at Austin with Dr. Christine Schmidt as an advisor. She obtained her M.S. degree in Chemical Engineering in 2004. Natalia is the author of six first-author publications and proceedings, and a second author of two more publications. She presented her graduate work at four national meetings. Natalia got engaged to her fiancé, Luis Eduardo in 2005.

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