Development of Patterned Self-Assembled Monolayers Toward the Study of Axonal Differentiation

Dawn Marie Johnson

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Development of Patterned Self-Assembled Monolayers Toward the Study of Axonal Differentiation

by

Dawn Marie Johnson

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2012

Saint Louis, Missouri
ABSTRACT

The following work discusses the development of several techniques and new methods for the production of patterned surfaces for protein and cell confinement. These well-defined substrates allow us to study the mechanism of axonal differentiation in neurons confined to a two-dimensional starburst pattern. We utilize self-assembled monolayer (SAM) chemistry in conjunction with microcontact printing to create stable patterned substrates for cell culture. Photolithography is employed in the fabrication of patterned masters, which are used to create elastomeric stamps for microcontact printing.

Initially, trichlorosilanes were employed in our patterned SAMs because they react rapidly with glass. These patterned surfaces confined protein and cells to a defined pattern; however, trichlorosilane monomers were difficult to work with because of their extreme reactivity with moisture in the air. An alternative to this highly sensitive system was required to develop stable SAMs. Alkanethiols on gold have traditionally been stable for just 5-7 days in cell culture, but modifications to the linkage between the alkane chain and glycol termination led to the formation of a stable self-assembled monolayer for over five weeks. This is a tremendous advance in the field of SAM chemistry and allows for the study of cellular processes that occur over the course of several weeks.

While long-term stability is necessary for the study of developmental events, there are many researchers who do not have the resources to fabricate their own patterned substrates. This led to the development of recyclable, reusable patterned SAMs for cell culture. By utilizing two different methods, either a trypsin analog or detergent, these substrates can be reused up to 11 times over the course of two weeks. This allows
investigators to perform several studies on the same patterned substrate, which leads to rapid, reproducible results.

The interesting biological question we set out to answer was whether axonal differentiation was an innate process or one that was environmentally determined. We cultured E18 mouse hippocampal neurons on starburst patterned substrates. The starburst consisted of twelve paths of equal width; eleven were short, 20 µm paths and one was longer, ranging from 40 µm to 160 µm. We observed which path the axon grew along by immunostaining for the microtubule-associated tau protein, bound to microtubules in the axon. Our data showed that the axon grows along the long path ~58% of the time for the smallest starburst pattern and the distance a neurite is allowed to extend down a path is linearly correlated to the likelihood of finding the axon on the long path. This points toward axonal differentiation being an environmentally determined process.

This combination of photolithography, microcontact printing, and self-assembled monolayer chemistry has led to important advances in the production of stable, patterned substrates for cell culture. We have successfully used this technology to study axonal differentiation and have found that this process is environmentally determined.
ACKNOWLEDGMENTS

First, I would like to thank my advisor, Joshua Maurer, for his constant support and encouragement. Anytime I needed help or a little motivational nudge, he was there. Whenever I was indecisive about something that seemed very important, like the color of plants for a fish tank, he reminded me that ‘these are the types of decisions you have to make now that you’re in grad school’. But on a more serious note, I am very lucky to have had someone like Josh to point me in the right direction these past six years. Thank you for taking the time and having the patience to help me whenever I needed it.

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To all my friends both in and out of graduate school, I am so thankful to have such wonderful people in my life. You have all been incredible sources of support and have filled my days and nights with laughter.

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the path to successful research. Thank you to Prof. Erik Herzog and Prof. Jacob Schaefer for agreeing to be a part of my defense committee; I’m looking forward to hearing your thoughts on my exciting research.

I have been fortunate enough to work on a project that was funded by the National Institute of Mental Health (1R01MH085495). I am thankful to have had financial support through the NIH these past few years.

On a personal note, I wouldn’t be here today without the loving support my family has given me all along. I want to thank my parents for always asking how my research is going and having a genuine interest in what I was doing in lab. I think I heard the phrase ‘but you’re almost finished, the hard part is behind you’ at least 100 times, starting in my third year. I think they figured that eventually it really would be. Well, you were right, as always. I am very lucky to have two optimists for parents. Thank you for believing in me every step of the way.

To my sister, Maggie, we have always chosen opposite paths—the best example would be chemistry vs. entrepreneurship. We tell each other all about what we do at school and don’t understand a single word of what the other one says, but we always support each other. Sissy, you make me smile every time we’re together and teach me something new every day.

During my time here at Washington University, I’ve gained a new family, Eric, Tricia, and Patrick, my FIL, MIL, and BIL. Many people dread seeing and spending time with their in-laws, but I count down the days until we are together. Thank you all for welcoming me into your family—long talks in the living room, cooking, and watching movies together are some of the times I look forward to most.
And of course, last but certainly not least, I want to thank my incredible husband, Ben.

Probably the most amazing part about this journey is that we have gone through it side-by-side. Two people under the same roof finishing their theses at the same time—and the house is still standing, unbelievable! I couldn’t have made it here without you. Thank you for being the most supportive, understanding, patient best friend. I am so incredibly lucky to have you in my life.
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<tr>
<td>3D</td>
<td>Three-Dimensional</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<tr>
<td>APTMS</td>
<td>Aminopropyltrimethoxysilane</td>
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<tr>
<td>CHO-K1</td>
<td>Chinese Hamster Ovary cell line</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>E18</td>
<td>Embryonic day 18</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FC</td>
<td>Fragment crystallizable region</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MEA</td>
<td>Microelectrode Array</td>
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<tr>
<td>MEMS</td>
<td>Microelectromechanical Systems</td>
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<td>N-Cadherin</td>
<td>Neural Cadherin</td>
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<tr>
<td>NF-H</td>
<td>Neurofilament-H protein</td>
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<tr>
<td>NIH/3T3</td>
<td>Mouse embryonic fibroblast cell line</td>
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<tr>
<td>PDMS</td>
<td>Polydimethyl siloxane</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PMGS</td>
<td>Plasma membrane ganglioside sialidase</td>
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<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<tr>
<td>QNM</td>
<td>Quantitative Nanomechanical Mapping</td>
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<td>SAM</td>
<td>Self-Assembled Monolayer</td>
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<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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</table>
SPM        Scanning Probe Microscopy
UV         Ultraviolet
CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

The ultimate goal of this body of work has been to develop patterned self-assembled monolayers (SAMs) for the study of cellular behavior in response to two- and quasi three-dimensional confinement. In order to achieve these results, a combination of chemistry and substrate preparation techniques have been employed. Photolithography was employed to fabricate all the necessary patterned masters for microcontact printing. Self-assembled monolayer chemistry allowed for short- and long-term protein and cell confinement as well as the production of reusable patterned substrates. In addition to these initial SAM development studies, we have been able to use these stable patterned SAMs to study axonal differentiation and to determine whether this cellular process is
predetermined or environmentally determined. A combination of organic chemistry, materials science, and cell biology have been employed to create well-defined patterned substrates; this allows us to take a closer look at neuronal behavior in constrained environments.

1.2 PHOTOLITHOGRAPHY

Photolithography is the process by which light is directed onto a substrate to produce a pattern. This technique is utilized heavily in the fields of computer chip fabrication, microelectromechanical systems (MEMS) devices, biosensors, and several other areas of research and technology.\(^1\)\(^5\) As in many other applications and for our purposes here, the pattern is formed in a light-sensitive polymer, known as photoresist, which is then further processed to create the desired product. Photoresist chemistry is known as chemical amplification and is a catalytic process initiated by photochemical acid generation. Typically, a triphenylsulfonium hexafluoroarsenic salt, as shown in Figure 1.2.1, is used as the photoacid generator. These types of sulphonium salts are designed to be thermally stable to temperatures greater that 200 °C and substitution of the phenyl rings can be used to change their spectral adsorption properties.\(^6\) When this triphenylsulfonium salt is irradiated, the sulfonium salt is decomposed and a proton is released. Two different types

![Figure 1.2.1. Triphenylsulfonium hexafluoroarsenic salt. Irradiation with ultraviolet light results in acid generation.](image-url)
of chemically amplified photoresists are commercially available, positive resist, which uses bond cleavage, and negative photoresist, which uses polymer crosslinking. One example of bond cleavage in a positive photoresist system is shown in Figure 1.2.2. Here, the cleavage of an ester bond results in a base soluble mixture of products. This, in turn, leads to pattern formation; irradiated positive photoresist is washed away during

**Figure 1.2.2.** Positive photoresist chemistry. The presence of an acid leads to disruption of the ester bond and formation of a base soluble product, a vinyl, and carbon dioxide.

**Figure 1.2.3.** Negative photoresist chemistry. The presence of an acid leads to polymerization of both monomer and polymer chains present in the photoresist.
development. In the case of negative photoresist, the proton generated leads to crosslinking of the polymer chains, as shown in Figure 1.2.3. In a chemical amplification system, a single event leads to a cascade effect causing widespread reaction throughout the photoresist layer. This leads to rapid, irreversible pattern formation. However, the reaction is also tightly controlled by outside factors during UV irradiation to prevent undesirable excess reactions. For example, the temperature and humidity are held constant within the cleanroom to provide water in the atmosphere, which will maintain consistent fabrication conditions. Upon irradiation of a photoresist with UV light, water is essential in the generation of an acid from the triphenylsulphonium salt shown in Figure 1.2.1. Excess water present during the reaction leads to increased reaction rates, which decreases one’s control over pattern formation. In contrast, low humidity causes slowed acid generation and therefore incomplete pattern fabrication.

The production of patterned substrates requires several pre-fabrication steps followed by post-exposure finishing steps. First, a substrate, often a polished silicon wafer, is cleaned in preparation of photoresist application. This varies according to the type of photoresist used. Positive resist requires an acetone wash to remove any particles present while negative resist requires a piranha etching step followed by dehydration of the wafer just prior to application of the resist to remove any organic impurities and water, which can hinder binding to the substrate. Photoresist is applied to the wafer using a spin-coater system at the desired speed and acceleration in order to achieve a uniform coating of the appropriate thickness; the faster the spin speed, the thinner the resist layer. The resist-coated wafer is then soft-baked to remove excess solvent from the resist, which can interfere with acid generation and subsequent bond cleavage or polymerization reactions.
during UV exposure. Next, the coated wafer is exposed to UV light, either passed through a chrome-patterned mask or directly using a direct-write system. After exposure, the resist-coated wafer will either go through a post-exposure bake step to complete crosslinking of the resist and binding to the substrate (negative photoresist only) or continue directly to developing. Once developing is complete, the patterned master can be utilized in future studies. The process of patterning with positive photoresist is shown

![Diagram of the fabrication process](image)

**Figure 1.2.4.** Schematic of the fabrication of a patterned master produced using positive photoresist and a mask aligner system.
Photoresist is a polymer that reacts upon exposure to a wide range of wavelengths of light.\textsuperscript{6,13} We utilize resist that is sensitive to ultraviolet (UV) light. The two main types of photoresist, positive and negative, are useful in different applications. Positive photoresist is mainly utilized in computer processing and production of biosensors consisting of multiple metals and layers,\textsuperscript{14-16} while negative photoresist is very useful in the production of MEMS devices and patterned masters for microfluidics.\textsuperscript{17,18} Both types of resist are commonly used to produce masters for soft lithography applications, depending on the desired feature size. Usually positive resists are less viscous than negative resists and are often easier to work with. However, negative resists are more stable in long-term applications due to their excellent chemical resistance.\textsuperscript{17} Another characteristic to note is the maximum working thickness of resist once it is spun onto a wafer. Most types of positive resist have a maximum thickness of 10-15 µm while negative resists can easily achieve thicknesses of over 100 µm.\textsuperscript{19} In addition, positive photoresists are able to achieve higher resolution, meaning smaller feature size, because the chemical amplification method of bond cleavage is more tightly controlled than the crosslinking reactions employed in negative photoresist.

As explained previously, when positive photoresist is exposed to UV light, ester bonds are cleaved and the irradiated resist becomes soluble during development with a basic solution. This leaves behind the desired pattern; however, the walls of the pattern are not vertical and residual resist is present along the edges. As a result, we can exploit this weakness and achieve smaller features by either under-exposing or under-developing the photoresist. In contrast, negative photoresist crosslinks upon exposure to UV light due to
acid-initiated polymerization reactions; non-crosslinked polymer is washed away during development with an organic solvent. The resulting pattern is the exact opposite of positive resist and 90° angles are present between the substrate and walls of the pattern as in Figure 1.2.5.

It is possible to carry out photolithography with several different types of instruments. Traditional photolithography involves the use of a chrome mask in conjunction with a mask aligner. A mercury lamp emitting a range of wavelengths is passed through a band pass filter with a center at ~365 nm, shone through a chrome-patterned glass mask in contact with a photoresist-coated wafer, and the photoresist is exposed for a defined length of time. The limitations of this process lie mainly in the mask. The resolution of the final master is dependent on the feature size of the mask; more specifically, in a contact photolithography system, the features of the mask and the resulting patterned master would be the exact same size. In addition, it is more difficult to troubleshoot pattern size and configuration parameters because a new mask must be made each time.

**Figure 1.2.5.** Positive (A) and negative (B) photoresist post-development. Positive resist is removed where UV-irradiated, leaving residual resist near the edges of the pattern. Negative photoresist is cross-linked, resulting in a pattern containing vertical right angles.
an alteration to the pattern is desired. Alternatives to this set-up involve the use of a projection system or phase-shifting mask, which will allow for greater variation in pattern size; these systems generally shrink the pattern shone onto a photoresist-coated substrate.\textsuperscript{20,21} Still, the limitation in traditional photolithography is the mask itself.

An alternative to photomask/contact photolithography that we utilize is direct-write lithography with a LaserWriter system. This system involves the use of several focusing optics to direct a 325 nm He-Cd laser beam through an objective and onto a UV-light sensitive substrate. This direct-write LaserWriter system can achieve sub-micron resolution and allows for the production of virtually any desired pattern down to 600 nm resolution. In addition, it is possible to write three-dimensional patterns through the use of grayscale lithography utilizing our LW325 LaserWriter system.\textsuperscript{22}

Our LaserWriter system writes a pattern in two primary ways: beam scan mode or stage scan mode.\textsuperscript{22} Beam scan mode utilizes a piezo-actuated mirror to raster the laser beam across the surface as the stage moves in the y direction. The acousto-optic modulator, in conjunction with the power meter, corrects for variations in laser power and generates the desired exposure dose across the substrate. This mode is also commonly used for grayscale lithography. Stage scan mode works by moving the stage in both the x and y directions to create a desired pattern. Here, the beam is not rastered across the substrate; it is held at a single position as the stage is moved. This mode is often employed when one needs a large number of photons to hit the substrate, as the stage can be moved very slowly, and is quite useful in photoablation studies.\textsuperscript{23}
Grayscale lithography allows for three-dimensional substrate fabrication. The laser is rasterized across the substrate, with the exposure dose determined by a grayscale bitmap file. While grayscale images have traditionally been produced in positive photoresist,\textsuperscript{24, 25} we have chosen to focus on working in negative photoresist due to the large variation in thicknesses as well as the increased stability of the substrate.\textsuperscript{17} Through our attempts at the fabrication of three-dimensional patterned substrates, we have found that the post-exposure bake causes the crosslinked polymer, now more dense, to sink and the non-crosslinked polymer to rise around the solid negative resist. This leads to the formation

\textbf{Figure 1.2.6.} Grayscale photolithography. Negative photoresist is crosslinked when exposed to UV light. During the post-exposure bake, the crosslinked, solid polymer (dashed lines) sinks to the substrate while the less dense, liquid resist (solid lines) rises to the surface. Developing results in a solid, 3D patterned substrate.
of a three dimensional substrate, as shown in Figure 1.2.6.

1.3 SELF-ASSEMBLED MONOLAYER CHEMISTRY

Self-assembled monolayer (SAM) chemistry provides the basis for the work described here. In order to form a SAM, a monomer consisting of a reactive head group, long alkane chain, and tail group, must first react with a substrate; subsequent monomers react with the substrate and organize tightly through van der Waals interactions and hydrophobic forces to form a monolayer (Figure 1.3.1). SAMs can be formed on a wide range of substrates from glass to metal oxides to gold.26-33 Numerous studies have been conducted to determine the physical properties and kinetics of self-assembled monolayers.34-38 Molecular simulations have also been employed to gain a better understanding of the dynamics of SAMs and the inevitable creation of defect sites within a monolayer.39,40

Perhaps the most well studied SAMs are alkanethiols on gold.41-48 While initial studies in our group focused on the assembly of silanes on glass,10,49 it was determined that well ordered, long-term self-assembled monolayers were difficult to produce and maintain. This was mainly due to the instability of the monomer itself upon exposure to air and because of the crosslinking between monomers on the substrate. The next scheme our group was interested in studying was a phosphonate on glass system. Phosphonates are less reactive than silanes and could potentially offer a more stable system due to
increased bonding to the substrate.\textsuperscript{50,51} However, a reliable, reproducible method for the production of phosphonate SAMs on glass was not achieved. Therefore, we turned back to thiols on gold to produce SAMs capable of resisting deterioration for long periods of time.\textsuperscript{52} Gold/thiol systems are a good choice for several reasons. Gold, although somewhat costly, is a stable, non-oxidative metal that is non-toxic to biological systems. In addition, thiols bind to gold with high affinity,\textsuperscript{53} displacing less tightly bound molecules on the surface. This, in turn, suggests that an alkanethiol on gold system could be stable for long periods of time. Also, gold reacts quickly with thiol head groups, however, organization of the monolayer may occur over several hours,\textsuperscript{54} creating a SAM with few internal defect sites.

While thiols on gold can be a reliable system for long-term stability studies, they are susceptible to several problems as well. First, the substrate cleanliness and ‘flatness’ of the gold play a large role in SAM order or disorder.\textsuperscript{55-57} When a gold substrate is rough, defect sites occur between monomers or cause islands to form.\textsuperscript{58} Other factors contributing to disorder or ill-formation of a self-assembled monolayer occur when bulky tail groups are present or when the initial monomer solution contains impurities, leading to a change in kinetics and often slowed SAM formation.\textsuperscript{32} In addition, thiol monomers can be displaced by other thiols or disulfides in solution over the course of several hours to days.\textsuperscript{59,60} This replacement process is more rapid at defect sites and areas of disorder. A final drawback associated with thiols on gold, which leads to disruption of the monolayer, is oxidation of the thiol head group, leading to displacement of the monomer.\textsuperscript{61}
1.4 **SOFT LITHOGRAPHY**

The ability to produce stable, patterned self-assembled monolayers is key to the study of confined cell growth. In 1995, George Whitesides and coworkers developed soft lithography, one application of this technology is widely known as microcontact printing.\(^{62, 63}\) This process uses an elastomeric stamp, produced by curing polydimethyl siloxane (PDMS) on a patterned master, as in Figure 1.4.1, to create a patterned SAM. The stamp is inked with hexadecanethiol, a long alkane chain monomer which will adsorb protein, and applied it to a gold substrate. After removing the stamp, the gold substrate is immersed in a glycol-terminated thiol solution to fill the background with a protein and cell resistant SAM. Figure 1.4.2 demonstrates how a patterned gold coverslip is produced by microcontact printing. For many years, patterned alkanethiols on gold were only stable for 5-7 days in cell culture.\(^{64, 65}\) With the incorporation of mannitol-terminated alkanethiols or a D+L gulitol racemic sugar system, SAM stability and protein/cell resistance was increased to 25 and 23 days, respectively.\(^{66, 67}\)
Our group has synthesized amide-linked glycol-terminated monomers capable of long-term stability of over five weeks in culture. This advancement allows for cellular studies to be conducted for several weeks rather than several days.

**Figure 1.4.2.** Production of a patterned gold coverslip. Hexadecanethiol, a protein adsorbant SAM, is microcontact printed onto a gold-coated coverslip and then soaked in glycol-terminated alkanethiol. The pattern is visualized by incubation with AlexaFluor 647 labeled fibronectin.
There are several other methods for patterned protein immobilization on a substrate. These include inkjet printing, polymer casting, and direct protein printing. Inkjet printing emerged in the late 1990s as a method for printing molecules, proteins, and other solutions. While the resolution is actually quite good and it is relatively simple to construct a print head for this type of patterning, the solution must be either heated to boiling or subjected to sonication with a piezoelectric device. High heat alone would prevent use with many proteins since they would denature at high temperatures and likely misfold upon cooling. Sonication is much more gentle but can result in protein shearing. Also, care must be taken to avoid dehydration of the protein on the substrate, which could lead to a change in conformation and decreased activity. Polymer casting is another method that would provide a substrate with several sites for protein immobilization (Figure 1.4.3). Zelma et al. used a combination of polymers to create domains; one of which is soluble in cyclohexane. This results in a substrate containing domains where protein can adsorb surrounded by non-adsorptive areas. Another group, Shimomura and co-workers, created honeycomb-patterned polymer films composed of amphiphilic copolymers containing lactose toward the end goal of cell culture. They observed that the natural honeycomb pattern of the thin film allowed for increased cell adhesion and growth whereas lack of a patterned thin film prevented cell attachment and outgrowth.

An alternative to these simple casting methods involves the use of photolithography,
microcontact printing, and other methods to pattern polymer films for cell culture. The final alternative patterning method discussed here is direct protein printing. In this method, protein is applied directly to a PDMS stamp, dried, and placed in contact with a substrate for an extended length of time. Often, pressure must be applied to the stamp to maintain contact with the surface. Direct printing of proteins may result in decreased activity of the protein due to denaturation on the substrate (Figure 1.4.4). Biasco et al. performed atomic force microscopy (AFM) studies to determine the size of metalloprotein structures after microcontact printing and found them to be 2-fold smaller than the native state. They attributed this to the variations in types of materials the protein encounters during microcontact printing, more specifically, the combination of hydrophobic/hydrophilic properties of the PDMS stamp and substrate. However, several groups have successfully patterned various proteins to study cellular behavior and activity. For example, Eichinger et al. developed a system for microcontact printing multiple proteins on a substrate. They wanted to mimic the natural protein environment a cell would experience in vivo. Using a live-cell imaging set-up, they were able to align multiple PDMS stamps and print independent proteins in a well-defined pattern. Jandt and co-workers patterned extracellular matrix (ECM) proteins onto a chitosan-coated substrate, which allowed for a more stable protein pattern. Osteoblasts cultured on these surfaces preferentially grow along the ECM pattern rather than the chitosan background.

**Figure 1.4.4.** Schematic of microcontact printing protein directly onto glass.
Microcontact printing has been applied to several other fields of study beyond patterned cell growth, including patterned lipid bilayer studies, fabrication of patterned magnetic nanoparticles, and creation of multi-color luminescent films for use in display devices, providing a wide range of applications for soft lithography.

1.5 Axonal Differentiation

Neurons are responsible for the vast majority of communication throughout the body, allowing messages to be transmitted from the brain to various organs, muscles, and all other parts of an organism. These signals initiate single events, which lead to cascade reactions or more complicated multi-step processes. Signals are sent by the axon and received by dendrites of another neuron. Typically, each neuron consists of a single axon and many dendrites, all working in unison to send and receive messages that will aid in the life cycle of the neuron and in turn, the organism itself. A crucial event in the development of a neuron is axonal differentiation. A better understanding of the inherent mechanism of neuronal polarization will lead to greater knowledge of how neuronal pathfinding is achieved in vivo.

Axonal differentiation is signified by the compartmentalization of key protein markers expressed by the cell. Microtubule-associated protein tau is a widely utilized axonal marker, expressed and bound along the microtubules of the axon. It coordinates with microtubules at the distal end of the axon, near the synapse. Microtubule-associated protein 2 (MAP2) is transported to the dendrites once polarization has occurred.

There are several stages leading up to neuronal maturity, depicted in Figure 1.5.1. In stage 1, the neuron is budding and beginning to develop lamellipodial and filopodial
protrusions. Stage 2 is achieved when the neurites start to grow more rapidly, but are still similar lengths. However, a single immature neurite may be growing slightly faster than the others. Once one neurite has grown past all others and axonal and dendritic markers have begun to segregate to their respective areas of the neuron, stage 3 has occurred. At stage 4 neurons continue to grow and branch, beginning to form neuronal networks. By the time a neuron reaches stage 5, approximately two weeks later, synapse formation occurs.

Much controversy has developed over whether axonal differentiation is an innate or environmentally determined process. Both sides have been argued with highly convincing studies. Abad-Rodriguez and co-workers and Dotti and co-workers found that neuronal polarization is a predetermined event coerced by a single protein, plasma membrane ganglioside sialidase (PMGS).\textsuperscript{93,94} They found that PMGS was expressed in the neurite destined to develop into the axon (Figure 1.5.2). Time-lapse microscopy, growth stunting and acceleration, as well as overexpression of the protein in addition to

\textbf{Figure 1.5.1}. Five stages of axonal differentiation. Stage 1 is observed at 0 days \textit{in vitro} (div), stage 2, extension of immature neurites, is observed 1-2 div, stage 3, extension of a single neurite past all others, the axon, is seen at 2-4 div, stage 4, increased axon and dendrite growth and branching, occurs 4-15 div, and stage 5, synapse formation is finalized, at 15-25 div.
several other assays were performed. In each study, they found overwhelming evidence that axonal differentiation is predetermined due to the localization of PMGS in a single neurite, beginning very shortly after cell culture. In contrast, a study by Pennypacker et al. found that the axonal marker neurofilament-H protein (NF-H) and MAP2 are co-localized early in development. This would suggest that neuronal polarization is not

Figure 1.5.2. Plasma membrane ganglioside sialidase (PMGS) localization. A developing neuron was found to have increased levels of PMGS in the neurite which would become the axon. Once differentiation occurred, PMGS remained localized in the axon.

Figure 1.5.3. Compartmentalization of axonal and dendritic markers. Tau, an axonal marker, and MAP2, a dendritic marker, are co-localized early in development, during stages 1 and 2. However, these markers are segregated to their respective locations by stage 3 of development.
predetermined, rather it is environmentally determined and these markers are segregated to the axon and dendrites as growth is occurring (Figure 1.5.3). In addition, several studies arguing for one or the other further fuel the desire to solve whether axonal differentiation is preprogrammed or environmentally determined.\textsuperscript{89,91,96-98}

1.6 SUMMARY

A wide variety of problems can be studied through the use of photolithography, soft lithography, and self-assembled monolayer chemistry, including protein and cell confinement studies, developmental events, and cellular behavior investigations. The work described in the following chapters provides initial substrate design as well as biological studies to aid in future work addressing the use of patterned systems to probe biological questions.
1.6 References


30


CHAPTER TWO

DIRECT PRINTING OF TRICHLOROSILANES ON GLASS FOR SELECTIVE PROTEIN ADSORPTION AND CELL GROWTH

2.1 INTRODUCTION

Here we describe simple methodology that allows for direct microcontact printing of octadecyltrichlorosilane onto glass coverslips followed by backfilling with an ethylene glycol-terminated trichloroalkane silane; this produces patterns with regions that promote and prevent protein adsorption and allow for control of cell growth.

Self-assembled monolayers (SAMs) formed from alkanethiols on gold have been used to produce surfaces that confine cells and proteins to well-defined patterns. These surfaces have proven to be useful for understanding a number of central biological processes and play critical roles in the formation of protein microarrays, drug discovery studies, and biosensor development. However, the methodology
employed in these studies requires the use of specialized equipment to prepare both the pattern and its substrate. While microcontact printing provides a rapid way to fabricate well-defined patterns without the need for complex lithography equipment, the need for an electron beam evaporated gold substrate impacts the simplicity of this method.

Traditionally, patterned substrates have been prepared by both classical photolithographic and microcontact printing methods. The simplest method involves transferring proteins to glass slides using microcontact printing. However, direct printing of protein onto a non-functionalized substrate often results in denaturation and possible decreased activity. More importantly, this system does not easily allow for the creation of protein resistant regions. The result is rapid, non-specific protein adsorption and cell growth covering the entire substrate, which makes it nearly impossible to track the activity of a particular area of cell growth over several days. To prevent this problem, Whitesides and co-workers have developed a well-defined alkanethiol self-assembled monolayer system. In this system, hexadecanethiol is stamped onto a gold coated coverslip and the background region is coated with an ethylene glycol-terminated alkanethiol, which forms a protein resistant SAM.

For the success of this methodology, substrate preparation is critical; it has been established that confinement is extremely sensitive to the preparation of the gold coating. For example, gold slides prepared by thermal evaporation give very poor confinement, while gold slides prepared by electron beam evaporation give good confinement. Typically, glycol-terminated alkanethiol SAMs confine cell growth
for approximately five days,\textsuperscript{12,13} however; it has recently been established by Luk and co-workers that the angle of electron beam evaporation can significantly improve the temporal stability of confinement.\textsuperscript{14} Additionally, the use of a gold surface can be deleterious for some biological studies. Optically transparent gold-coated coverslips can be prepared for live cell microscopy on an inverted microscope; however, these slides serve as a neutral density filter, reducing light to the camera. Gold is also known to be a fluorescence quencher, which can reduce the efficiency of fluorescence-based studies.\textsuperscript{15,16} Since gold is a short-range quencher, it can greatly effect visualization of fluorescently labeled proteins adsorbed to a surface.

\subsection*{2.2 RESULTS AND DISCUSSION}

To combat these problems, we have developed methodology to directly pattern glass surfaces using SAMs prepared from trichlorosilane terminated monomers. Previous studies demonstrated that patterning of SAMs formed from octadecyltrichlorosilane (1) by UV ablation followed by protein adsorption resulted in preferential adsorption of the protein on the hydrophobic octadecylsilane monolayer.\textsuperscript{17} Additionally, it has been shown that octadecyltrichlorosilane can easily be patterned by microcontact printing.\textsuperscript{18} However, bare glass does not block protein adsorption onto the surface.

\begin{figure}[h]
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\caption{Octadecyltrichlorosilane (1) and triethylene glycol-terminated alkane silane (2).}
\end{figure}
Therefore, we developed a molecule which would block protein adsorption and allow for the production of a patterned surface.

Based on the work of Prime and Whitesides, we anticipated that an ethylene glycol-terminated silane would prevent nonspecific protein adsorption. As a result, we synthesized triethylene glycol-terminated alkane silane (2). The synthesis of 2 was carried out by the formation of an alkene terminated glycol from commercially available starting materials. The resulting alkene was converted to triethylene glycol-terminated alkane silane using chloroplatinic acid as a catalyst. The reaction of 2 (as a 5 mM solution in toluene) with glass resulted in monolayer formation. These monolayers block non-specific protein adsorption and prevent cell growth.

To prepare patterns using trichloroalkane silanes we employed a microcontact printing approach. The general strategy was similar to that which has previously been used for alkanethiols on gold and is illustrated in Figure 2.2.2. Octadecyltrichlorosilane (1), as a 10mM solution in toluene, was inked onto a PDMS stamp which was stamped onto a clean glass substrate. A glycol-terminated monolayer was then formed on the bare regions of the glass by immersing the substrate in a solution of 2. Fluorescently labeled fibronectin was then allowed to adsorb to the pattern and cells were plated onto the substrate.

To develop robust methodology, we looked at stamping and backfilling as a function of time. We found that the protein resistant ethylene glycol-terminated SAMs formed rapidly (in less than 30 minutes) from a 1 mM solution. Additionally, increasing the soaking time neither enhanced nor retarded the ability of the resulting monolayer to resist nonspecific protein adsorption. This is in contrast to the formation of ethylene
glycol-terminated SAMs on gold, which require 12-14 hours.\textsuperscript{12} Formation of trichlorosilane monolayers is most likely faster due to the high reactivity of the trichlorosilane group with glass surfaces.

While ethylene glycol-terminated monolayer formation is not influenced by soaking

\textbf{Figure 2.2.2.} Illustration of the stamping methodology employed to directly pattern protein adhesive and non-adhesive regions on a glass substrate.
time, significant differences were observed for stamping times longer than 15 seconds. Stamping for less than 15 seconds gave well-defined patterns as visualized by the adsorption of anti-guinea pig IgG FITC conjugate (Figure 2.2.3). Stamping times that were longer than 15 seconds resulted in smearing out of the pattern. This is not unexpected given that the octadecyltrichlorosilane molecule diffuses out from the stamp before reacting with the surface. In addition, at long stamping times, bright rings are observed around the edge of the pattern. This is likely a result of multilayer

![Figure 2.2.3](image)

**Figure 2.2.3.** Varying stamp contact times followed by selective adsorption of anti-guinea pig IgG FITC conjugate onto a patterned trichlorosilane surface.
formation in these regions as a greater amount of octadecyltrichlorosilane would be present at the edges of the stamp due to its vertical features. The observation of increased protein fluorescence is then simply due to the increase in hydrophobic surface area resulting from the multilayers.

Since significant variations were seen with stamping time, we also explored varying the concentration of the octadecyltrichlorosilane ink. For short stamping times (5 seconds), no visible differences in pattern sharpness were observed using ink

![Image of CHO-K1 cell growth on a fibronectin coated trichlorosilane surface.](image)

**Figure 2.2.4.** Selective CHO-K1 cell growth on a fibronectin coated trichlorosilane surface. (a-b) Fluorescent image of fibronectin tagged with Alexa Fluor 647 adsorbed to the surface. (c-d) Phase contrast image of cell growth within the pattern. (e-f) Overlay of the fluorescent and phase contrast images.
concentrations of 1, 10, and 100 mM. Taken together with our stamping time results, this suggests a monolayer is formed at short stamping times and multilayers are formed at longer stamping times, regardless of concentration. Since trichlorosilane is highly reactive with ambient moisture, it is not surprising that multilayers are formed during long stamping times in air.

Having optimized the method using anti-guinea pig IgG FITC conjugate, we examined the adsorption of fluorescent fibronectin onto a patterned substrate. Fibronectin forms well-defined protein patterns that allow for selective CHO-K1 cell attachment and growth (Figure 2.2.4). CHO-K1 cells are quite robust; they attach non-specifically and grow on a wide variety of materials including glass, plastic, and adhesion proteins. Thus, they provide a good metric to judge the ability of the glycol-terminated SAM to prevent protein adsorption and cell growth. In our system, when CHO-K1 cells are introduced to a patterned surface, as described above, the cells grow within the pattern; their shape and direction of growth are manipulated by the octadecylsilane and triethylene glycol-terminated alkane silane SAMs.

2.3 CONCLUSIONS

Microcontact printing of octadecyltrichlorosilane followed by backfilling with an ethylene glycol-terminated silane monolayer is a broadly applicable approach to patterning a wide range of biological molecules. Furthermore, the protein resistance resulting from ethylene glycol-terminated silane monolayers is not specific; rather it can be applied to any protein and pattern combination. The use of trichlorosilane terminated molecules on glass provides a fast, efficient method for patterning biological materials on a surface. Moreover, this methodology has several advantages
over the classic alkanethiol system in that patterns can be formed directly on glass surfaces.
2.4 MATERIALS AND METHODS

All reactions were carried out under an atmosphere of dry argon and commercial solvents and reagents were used without further purification, unless otherwise indicated. Dry tetrahydrofuran (THF) was obtained from J. T. Baker in CYCLE-TAINERS. NMR spectra were collected on a Varian UNITY 300 spectrometer. Photolithography was carried out using a HTG-3HR mask aligner with near UV optics and a CEE 100cb spin/bake system. Fluorescent and phase contrast images were obtained using a Nikon TE2000-PFS microscope running NIS-Elements imaging software and equipped with a Prior XY stage, EXFO X-Cite series 120PC UV illuminator, Photometrics CoolSNAP monochrome camera, and In Vivo Scientific incubation system.

2.4.1 PDMS Stamp Preparation

Master Formation. A virgin silicon wafer (50mm, Montco Silicon) was treated with piranha solution (7:4 concentrated sulfuric acid:30% hydrogen peroxide) for 2 h, rinsed thoroughly with deionized water, and dried with nitrogen. Safety Note: Use extreme caution when working with piranha solution; it is highly corrosive and most likely explosive when in contact with organic solvents. SU-8 2015 negative photoresist (1-2 mL, MicroChem) was applied to the wafer and an even coating of resist (nominally 20 µm) was achieved using the six cycle spin-coater program shown in Table 2.4.1. Edge bead remover (7:3 THF:propylene glycol monomethyl ether acetate) was applied during the third and fourth cycles. The wafer was soft baked at 95 ºC for 5 m. Patterning was achieved by exposure on a mask aligner in hard contact mode for 180.0 s using a soda lime/chrome photomask (PhotoPlot Store). The wafer was post exposure baked for 6 m
at 95 °C and developed in propylene glycol monomethyl ether acetate (Sigma-Aldrich) for 20 s. The resulting master was hard baked at 180 °C for 3 h prior to stamp formation.

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**Table 2.4.1.** Spin-coater parameters for SU-8 2015 photoresist.

**PDMS Stamp Formation.** Sylgard 182 (Dow Corning) was mixed 10:1 (resin:hardener) and poured over the patterned silicon master. The polymer was degassed using a vacuum dessicator and cured at 70 °C for 2 h. The final stamp was separated from the master and cut to size.

**2.4.2 Synthesis of Oligo-PEG Terminated Trichloroalkanesilane**

![Chemical structure](image)

**11-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)undec-1-ene 3:** A solution of 11-Bromo-1-undecene (1.90 mL, 8.576 mmol, Sigma-Aldrich), triethylene glycol monomethyl ether (1.60 mL, 10.29 mmol, 1.2 eq, Fluka), 50% sodium hydroxide (1.30 mL, 17.15 mmol, 2 eq, Sigma-Aldrich) in THF (25 mL) was heated to reflux overnight. The reaction was diluted with deionized water (5 mL), extracted with hexanes (2 x 20 mL), dried over sodium sulfate, and concentrated *in vacuo*. The crude compound was subjected to flash column chromatography (3:1 hexanes:ethyl acetate) to give pure 3 (0.9033 g, 49.55%) as...
a colorless oil. $^1$H NMR (CDCl$_3$) d 1.25 (m, 10), 1.40 (m, 2), 1.60 (m, 2) 2.08 (m, 2), 3.40 (s, 3), 3.44 (t, 2), 3.65 (m, 12), 4.95 (d, 1), 5.00 (d, 1), 5.85 (m, 1).

11-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)undecyl)trichlorosilane 2: To 3 (0.1393 g, 0.316 mmol) and chloroplatinic acid hexahydrate (0.0138 g, 0.0316 mmol, 0.10 eq, Sigma-Aldrich), trichlorosilane (0.09 mL, 0.632 mmol, 2 eq, Sigma-Aldrich) was added in THF (10 mL). The reaction was allowed to proceed at room temperature until turning clear. The solvent was removed in vacuo and the crude product purified by Kugelrohr distillation (Buchi GKR-50) to give 2 (0.0307 g, 38.61%) as a colorless oil. $^1$H NMR (CDCl$_3$) d 1.25 (m, 12), 1.40 (m, 2), 1.65 (m, 2), 1.90 (m, 2), 2.00 (m, 2), 3.40 (s, 3), 3.44 (t, 2), 3.65 (m, 12).

2.4.3 Patterning Adhesive and Non-Adhesive Protein Regions on Glass

The stamping method described is given as an example; variations in the procedure are described below. Glass coverslips (25mm, VWR) were cleaned by soaking in piranha solution for 2 h. Coverslips were then twice rinsed with deionized water, dried under nitrogen, rinsed with ethanol, and dried under nitrogen. The stamp was coated with octadecyltrichlorosilane (1) (10 mM in toluene) by dropping the solution onto the stamp (5-6 drops) and drying with nitrogen. Slides were then stamped for 10 s. The bare regions of the glass were then allowed to react with a 1 mM or 5 mM 2 in toluene under argon for 0.5 h. After soaking, coverslips were twice rinsed with ethanol and dried under nitrogen.

Both stamping parameters and the reaction of 2 with the glass surface were explored in this study. Incubation in 1 mM 2, following stamping as described above, was carried
out for varying lengths of time: 5 m, 15 m, 30 m, 45 m, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 6 h, 8 h, and 10 h. The concentration of 1 used for stamping was varied: 1 mM, 10 mM, and 100 mM. A variety of stamping times were explored: 5 s, 10 s, 15 s, 30 s, 45 s, and 3 m (these were incubated in 5 mM 2).

2.4.4 Pattern Visualization

A patterned coverslip was placed in Attofluor cell chamber (Invitrogen) and rinsed three times with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen). The coverslip was then incubated with 5 µL/mL Anti-Guinea Pig IgG (whole molecule) FITC conjugate (Sigma) in DPBS at 37 °C for 1 h. Excess protein was removed by rinsing with DPBS (3x) and the coverslip was covered with fresh DPBS. Protein fluorescence was visualized using a Nikon B-2E/C filter cube.

2.4.5 Patterned Cell Growth

Preparation of Fluorescently Labeled Fibronectin. To 20 µL Human Plasma Fibronectin (1 mg/mL in 100 mM CAPS, 0.15 M NaCl, 1 mM calcium chloride, pH 11.5, Invitrogen) was added 1 µL of 1 M sodium bicarbonate in sterile water and 2 µL Alexa Fluor 350 carboxylic acid, succinimidyld ester (5 mg/mL in DMF, Invitrogen) or 2 µL Alexa Fluor 647 carboxylic acid, succinimidyld ester (5 mg/mL in DMF, Invitrogen). The reaction was mixed and allowed to proceed at room temperature for 1 h. The reaction was stopped by addition of 3 µL 1.5 M hydroxylamine in 1 N sodium hydroxide and mixed with 20 µL of unlabeled fibronectin.
**Cell Culture.** A patterned coverslip (stamped with 10 mM 1 and incubated in 5 mM 2 for 30 min) in either an Attofluor cell chamber or a small tissue culture dish was coated with fibronectin at 20 µg/mL as previously described for Anti-Guinea Pig IgG FITC conjugate. CHO-K1 cells (ATCC) were separated using TrypLE Express (Invitrogen), resuspend in Dulbecco’s Modified Eagle Medium (DMEM, low glucose 1X, glutamax, 1 g/L D-glucose, 110 mg/L sodium pyruvate, 50 mL FBS, 5 mL pen/strep, Invitrogen), and counted using a hemacytometer (Bright-Line, Hausser Scientific). After rinsing the patterned coverslip with DPBS, approximately 90,000 cells were applied in 1mL of DMEM. Plated cells were grown at 37 °C, 5% CO₂, and 96% RH). Cultures were visualized by inverted microscopy in an Attofluor cell chamber using phase contrast optics and either a Nikon UV-2E/C filter cube or a Semrock CY5-4040A filter cube.
2.5 References


CHAPTER THREE

INCREASED STABILITY OF GLYCOL-TERMINATED SELF-ASSEMBLED MONOLAYERS FOR LONG-TERM PATTERNED CELL CULTURE

Collaboration with Matthew K. Strulson*

3.1 INTRODUCTION

Classical systems for patterned cell culture, including self-assembled monolayers (SAMs) formed from alkanethiols on gold, have limited stability under cell culture conditions. Most systems are only stable for 5-7 days in cell culture,\(^1,2\) which significantly limits their use for the study of developmental events, in vitro disease models, and for long-term model systems for drug discovery.\(^3\)\(^7\) Monolayer instability has limited the use of patterned substrates to short-term cell culture experiments lasting only 1-2 days.\(^1,2,8,9\)

Here we develop a system that is stable for over five weeks in culture and explore the mechanism of SAM degradation, which has been of some debate.

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* MKS synthesized monomers and carried out SPM studies. DMJ performed long-term cell culture analysis. Both MKS & DMJ contributed to all data interpretation.
While the traditional ethylene glycol-terminated SAM monolayer (Figure 3.1.1a) is only stable for 5-7 days, a number of other systems with increased stability have been developed. These systems are typically based on sugar-terminated monomers and include the mannitol system developed by Mrksich and co-workers, which is stable for 25 days and the D+L gulitol racemic sugar system developed by Luk and co-workers which is stable for 23 days.\textsuperscript{2,10} Additionally, trichlorosilanes have been shown to form stable SAMs on glass for cell patterning,\textsuperscript{11} but the instability of these glycol monomers, which polymerize upon exposure to moisture, makes monolayer preparation notably more difficult than monolayer formation from alkanethiols on gold.

SAMs formed from alkanethiols on gold have been hypothesized to deteriorate due to several intrinsic and environmental factors, including: interfacial mixing of the monomers, blooming of the adlayer, and oxidation of the thiol head group. Whitesides and co-workers showed that patterned bovine capillary endothelial cells lose confinement by growing into the interface of the pattern.\textsuperscript{1} This loss of confinement was attributed to mixing of the hexadecanethiol and glycol-terminated monomers at the edges of the

![Figure 3.1.1.](image)

**Figure 3.1.1.** Protein and cell resistant SAMs were created from a) ether-linked glycol thiol (\textbf{1}), b) ester-linked glycol thiol (\textbf{2}), and c) amide-linked glycol thiol (\textbf{3}).
pattern through thiol migration, resulting in poor glycol coverage. An additional factor that has been hypothesized to affect monolayer stability is blooming. In blooming, the metal adlayer, which is required for the formation of gold-coated glass, alloys with the gold resulting in disruption of the monolayer.\textsuperscript{12-15} Moreover, the optically transparent thin gold films used in cellular studies were expected to be highly prone to blooming because the gold layer is extremely thin (typically 100 to 250 Å). Another factor hypothesized to contribute to SAM degradation is oxidation of the gold-sulfur bond to a sulfonate, which is unable to form stable covalent bonds to gold. Sulfonate formation has been measured directly by x-ray photoelectron spectroscopy and indirectly through increased stability of SAMs in deoxygenated media.\textsuperscript{16,17}

Here we demonstrate that we can dramatically increase patterned monolayer stability in cell culture by simply altering how the glycol moiety is attached to the alkanethiol (Figure 3.1.1). Cooper and Leggett previously reported that hydrogen bonding at the terminus of a SAM increased the stability of alkanethiol monomers to surface displacement.\textsuperscript{18} Also, the synthesis of a series of amide-linked glycol monomers and ester-linked glycol monomers have been reported and thermal stability of the SAMs was found to be dependant on the glycol-alkane chain linkage as evidenced by temperature-programmed desorption (TPD).\textsuperscript{19,20} However, ester and amide-linked glycol-terminated SAMs have not been studied under cell culture conditions. We demonstrate that ester and amide linkages greatly enhance patterned monolayer stability with the amide-linked monomer being stable on 100 Å gold for over five weeks in culture. The enhanced stability is due to the glycol-alkane chain linkage and not differences in van der Waal’s packing forces, since the monomers used in our study have the same number of
methylene units. Additionally, using quantitative nanomechanical mapping (QNM), we demonstrate that there is no substantial interphase mixing for any of the glycol-terminated monolayers. By looking at a variety of gold thicknesses, we demonstrate that blooming does not substantially affect monolayer stability in cell culture. However, we observe significant differences in monolayer stability as a function of gold thickness, which can be attributed to gold topology.

### 3.2 RESULTS AND DISCUSSION

The monomers utilized in these studies vary only in the linkage between the alkane chain and glycol moiety to rule out differences in glycol ordering and structure as factors that

![Figure 3.2.1. Synthetic scheme for the synthesis of the ester-linked glycol-terminated thiol (2).](image)

![Figure 3.2.2. Synthetic scheme for the synthesis of the amide-linked glycol-terminated thiol (3).](image)
affect protein resistance. Based on previous work, the tetraethylene glycol moiety should provide the necessary disorder in glycol structure to prevent protein and cell adhesion.\textsuperscript{21} The ether-linked monomer was synthesized as previously described.\textsuperscript{1,22,23} The synthesis of both the ester-linked and amide-linked monomers is straightforward from commercially available starting material (Figures 3.2.1 and 3.2.2). These syntheses are not significantly more onerous than that of the ether-linked monomers.

Patterns for cell culture were prepared by microcontact printing circles of hexadecanethiol onto gold substrates of varying thicknesses, backfilling with glycol-terminated monomers and non-specifically adsorbing fibronectin onto the hexadecanethiol-coated region.\textsuperscript{24} Gold thicknesses ranging from 50 Å to 250 Å with a 50 Å titanium adhesion layer in all cases were examined. These thicknesses were compatible with inverted live-cell phase-contrast microscopy. Thicker metal substrates introduced a substantial neutral density filter into the microscope and were thus not well suited for inverted microscopy.

To determine pattern stability under cell culture conditions, chinese hamster ovary (CHO-K1) cells were seeded onto fibronectin-coated substrates. CHO-K1 cells were chosen because they rapidly reach confluence and after becoming confluent daughter cells can detach and reattach in defect sites formed on the surface. As a result, stability experiments carried out using CHO-K1 cells, as opposed to a more slowly growing fibroblast cell line, such as NIH-3T3 cells, most likely represent a worst-case scenario for pattern stability. This is important both for understanding the mechanism of pattern degradation and defining cell culture stability. It is possible that previous studies, which have employed slow growing fibroblasts, have over-estimated pattern stability.\textsuperscript{2,10}
Figure 3.2.3. Live-cell phase-contrast images acquired weekly for CHO-K1 cells grown on a 95 µm circles pattern with an ether-linked glycol (1) monolayer background on varying gold thicknesses. Pattern stability is maintained for 14 days on 50 Å and 100 Å gold substrates.

Figure 3.2.4. Live-cell phase-contrast images acquired weekly for CHO-K1 cells grown on a 95 µm circles pattern with an ester-linked glycol (2) monolayer background on varying gold thicknesses. Pattern stability is maintained for 28 days on 100 Å gold substrates.
In order to monitor pattern integrity, substrates were imaged weekly until patterns reached approximately 50% confluence. Figures 3.2.3 through 3.2.5 show representative images of each gold thickness as a function of time for the three different glycol-terminated background monolayers (Figure 3.1). As is clearly seen in these images, pattern integrity is best maintained with the amide-linked monomer, followed by the ester-linked monomer, and finally the ether-linked monomer. This trend is in agreement

**Figure 3.2.5.** Live-cell phase-contrast images acquired weekly for CHO-K1 cells grown on a 95 μm circles pattern with an amide-linked glycol (3) monolayer background on varying gold thicknesses. Pattern stability is maintained for 35 days on 100 Å gold substrates.
with the thermal stabilities previously measured for these molecules.\textsuperscript{19,20}

It is important to note that the synthetic method employed for the formation of the ester-linked monomer is critical to monolayer stability. In initial experiments conducted using the ester-linked monomer prepared with a trityl protecting group, rapid pattern degradation was observed for samples prepared with background ester-linked monolayers. This degradation was likely due to trace acid-terminated monomers produced during the trityl deprotection, which in turn catalyzed ester hydrolysis. However, we were able to completely eliminate this instability by protecting the monomer as a disulfide (Figure 3.2.1).

A clear trend in pattern fidelity is also observed as a function of gold thickness for thicknesses between 100 Å and 250 Å. Surprisingly, this trend is the opposite of what would be predicted if blooming played a major role in monolayer degradation. If blooming was important to pattern instability, one would expect alloying to occur more slowly as the gold thickness increased and thus pattern stability to increase with increasing gold thickness. Here we observe the opposite trend for gold thicknesses between 100 and 250 Å. However, blooming may contribute to degradation of the SAMs formed on 50 Å of gold, since none of the patterned SAMs at this thickness confine cells longer than 14 days.

In order to quantitate the number of cells found in the background of the pattern, the number of spread (live, proliferating) and round (dead or weakly attached) cells growing outside the 95µm circle pattern were determined from 42-49 images obtained from three independent samples at each condition. Figure 3.2.6 shows the number of round and
spread cells for each glycol-terminated monolayer at each gold thickness. Substrates were considered confluent or partially confluent when the concentration of cells was greater than 200 cells/mm² this is indicated in the figure by a dotted line going off scale. We have found that often round cells are easily removed by thorough rinsing of the substrate and are not indicative of monolayer degradation.

For the ether-linked monolayer, a low number of background cells is observed until confluence. This implies that degradation of the ether-linked monolayer is a rapid process. The deterioration of both the ester-linked and amide-linked monolayers is more gradual than for the ether-linked monolayer. As a result, it is likely that the formation of defect sites in the ether-linked monolayer results in fast deterioration of the SAM, whereas the ester and amide-linked SAMs are able to maintain confinement in the presence of defect sites.

An interesting finding in this study is the increased stability of 100 Å gold substrates compared to 150 Å gold substrates. Traditionally, little attention has been paid to the substrate thickness used in patterned cell studies with typical gold thickness ranging from 120 Å to 2000 Å.¹,²,⁸-¹⁰,²⁵ However, our data suggests that gold thickness is a critical parameter in stability with 100 Å gold substrates providing increased stability relative to thicker and thinner substrates. Additionally, 100 Å gold substrates are beneficial, compared to thicker gold substrates, in studies utilizing epifluorescence microscopy, since the gold substrate acts as a neutral density filter, decreasing the light that reaches the camera. Moreover, it is possible to use 50 Å titanium/50 Å gold coated coverlips for short experiments (on the order of one week), which should provide even better fluorescence signals.
While Whitesides and co-workers observed pattern degradation by loss of confinement at the interface of the hexadecanethiol region and the glycol region,\textsuperscript{1} we do not observe cells

**Figure 3.2.6.** Cell attachment as a function of monomer linkage, gold thickness, and time. Spread cells are indicative of loss of pattern stability (A, C, E). Round cells are often unattached or weakly attached to the substrate (B, D, F). Dashed lines represent confluent substrates and complete loss of pattern.
growing out from the pattern edges. Instead, we observe cells attaching and spreading throughout the background region during loss of confinement (Figures 3.2.3-3.2.5). Whitesides and co-workers’ observation of cells growing out from the pattern is likely a result of using slowly replicating fibroblast cells, which do not readily detach and reattach at background defect sites. Our observation is consistent with sulfur oxidation and monomer loss as opposed to interfacial mixing as the mechanism of background monolayer destruction.\textsuperscript{16,17,26,27} While oxidation of the glycol moiety has previously been discussed,\textsuperscript{2,28} this is likely not the mechanism at play here since solvent accessibility, and therefore oxygen exposure, to the glycol moieties should be similar.

To further support our hypothesis that interfacial mixing is not a major contributor to glycol monolayer degradation, we examined interfacial mixing using scanning probe microscopy (SPM). While differences in hexadecanethiol versus glycol-terminated thiol regions of monolayers can be resolved in frictional force contact mode scanning probe microscopy,\textsuperscript{29} the observed height differences are likely artifactual. The observed height difference is likely due to significant differences in silicon tip adhesion between glycol-terminated and hexadecanethiol monomers. To examine interfacial mixing, we directly measured differences in tip adhesion as a function of time using QNM scanning probe microscopy (Figure 3.2.7). Samples for QNM analysis were prepared by microcontact printing 10 µm hexadecanethiol lines onto 150 Å gold-coated coverslips and backfilling with each of the glycol-terminated monomers. Force images were acquired weekly over three weeks for samples immersed in phosphate buffered saline at 37 °C. Despite significant differences of pattern fidelity in cell culture for some of these substrates, no significant changes were observed by force microscopy. If interfacial mixing were an
important part of monolayer degradation, we would have expected to see a blurring of the glycol/hexadecanethiol monolayer interface with time and differences between the three glycol monomers. However, the glycol-hexadecanethiol interface appears sharp in all samples after 21 days. It is interesting to note that while significant differences in

![Image](image_url)

**Figure 3.2.7.** Patterned substrate adhesion measured using QNM SPM. The wider lines are the glycol-terminated areas whereas the thinner lines are alkane-terminated. The ether-linked at day 1 (A) and day 21 (B), ester-linked at day 1 (C) and day 21 (D), and amide-linked at day 1 (E) and day 21 (F) do not show significant blurring of the pattern, indicating that interfacial mixing has not occurred.
adhesion and other mechanical properties between the hexadecanethiol and glycol regions were observed for samples immersed in phosphate buffered saline and washed with distilled water prior to measurement, no differences in adhesion or mechanical properties were observed if samples had been immersed in cell culture media without fetal bovine serum prior to measurement. This was true even when care was taken to completely rinse the substrate with distilled water prior to bringing it through the air/water interface.

To better understand the observed trend for gold thickness, we examine the roughness of the gold substrates using scanning probe microscopy in peak-force tapping mode (Figure 3.2.8). There are significant differences in appearance for the substrates with increasing roughness across the series from 50 Å to 250 Å. The change in roughness likely leads to a decrease in monolayer order, which in turn gives rise to the observed trend of decreasing stability with increasing gold thickness. Interestingly, the structure of the 50 Å substrate is very different from the other thicknesses and contains well-defined

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**Figure 3.2.8.** Scanning probe microscopy height images of gold substrates obtained in peak force tapping mode of 50-250 Å gold substrates (A-E).
nanostructures. These nanostructures are a result of the underlying titanium-coated glass coverslip and explain the limited stability of glycol-terminated monolayers on 50 Å gold. The 100 Å and 150 Å gold substrates resemble each other and consist of soft rolling hills, which likely support well-ordered monolayers. In contrast, the 200 Å and 250 Å substrates contain sharper “peaks and “valleys”. As a result, it is not surprising that the 100 Å and 150 Å substrates provide the best stability. Moreover, the 100 Å substrate, which contains more “hills” than “valleys”, is most stable. The observation that gold topology greatly affects monolayer stability is to be expected in light of the observations that increased pattern stability could be achieved by varying the angle of electron beam evaporation. However, unlike variable angle deposition, thickness control provides a readily available method for stability control. All commercially available electron beam evaporators can easily control substrate thickness, however most evaporators are not equipped for angular deposition.

3.3 CONCLUSIONS

Patterned SAMs with amide-linked glycol background monolayers prepared on glass coverslips with 50 Å of titanium and 100 Å of gold allow for more than five weeks of high-fidelity patterned cell culture. This represents an enormous advancement in patterned cell culture substrate stability and will allow for long-term cell culture experiments. We have also found that gold thickness can be used to control gold nanotopology and, in turn, monolayer stability under cell culture conditions. Furthermore, loss of pattern fidelity in cell culture does not arise from blooming or
interfacial mixing of the glycol monolayer with the hexadecanethiol monolayer and is therefore likely a result of sulfur oxidation and monolayer degradation.
3.4 MATERIALS AND METHODS

All reagents were obtained from commercial sources and used without further purification. Reactions were carried out in an argon atmosphere with dry solvents unless otherwise noted. $^1$H NMR and $^{13}$C NMR spectra were obtained on a 300 MHz Varian Innova instrument. Electrospray ionization mass spectrometry were obtained on either a Bruker Maxis Q-TOF or a Thermo LCQ Deca XP+, samples were dissolved in acetonitrile. Electron beam deposition was achieved using a PVD 75 electron beam evaporator (Kurt J. Lesker, Clairton, PA). Plasma oxidation was carried out in a Femto standard low-pressure plasma system (Diener electronic GmbH+Co. KG, Nagold). Live-cell phase-contrast images were obtained using a Nikon TE2000-PFS microscope running NIS-Elements imaging software and equipped with a Prior XY stage, Photometrics CoolSNAP monochrome camera, and In Vivo Scientific incubation system.

3.4.1 Synthesis of Ester-linked glycol thiol (2)

$12$-thioacetatedodecanoic acid (4) $12$-Bromohexadecanoic acid (1.00 g, 3.6 mmol) was dissolved in dimethylformamide (DMF) (20 ml) at 0 °C. Potassium thioacetate (1.00 g, 9 mmol) was added as a solid, turning the solution a deep red color, and the reaction was allowed to proceed for 30 minutes. The reaction was diluted with methylene chloride (50 ml) and washed three times with water (30 ml). The organic layer was dried over anhydrous magnesium sulfate. Followed by coevaporation of the DMF with toluene. The resulting material was purified by silica chromatography (toluene/ethyl acetate 20:1) to afford the product as a white solid. Yield: 0.604g (61.2%). Mp 64 °C. $^1$H NMR (300 MHz, CDCl$_3$): δ 1.3-1.33 ppm (14 H, s), 1.6 (4 H, m), 2.36 (3 H, s), 2.38 (2 H, t), 2.90 (2
H, t). $^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$ 24.95, 29.09, 29.33, 29.40, 29.45, 29.73, 30.96, 34.26. MS (ESI+) m/z calculated for C$_{14}$H$_{26}$O$_3$ +Na 297.1495, found 297.1509.

**Dodecanoic acid disulfide (5)** 12-Thioacetatedodecanoic acid, 4 (0.4211 g, 1.5 mmol) was dissolved in methanol (15 ml) and 25% sodium methoxide in methanol (3 ml). Air was bubbled through the reaction for 24 hours; the reaction was neutralized by addition of HCl, and diluted with methylene chloride (50 ml). The organic layer was washed 3 times with deionized water (60 ml) and dried over sodium sulfate. Concentration by rotary evaporation afforded the product as a white solid. Yield: 0.306 g (86.2%). Mp 83 °C. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 1.25-1.32 ppm (28 H, m), 1.64-1.7 ppm (8 H, m), 2.38 ppm (4 H, t), 2.72 ppm (4 H, t). $^{13}$C NMR (300 MHz, CDCl$_3$) $\delta$ 24.88, 28.59, 28.74, 29.21, 29.27, 29.41, 29.43, 29.55, 29.60, 29.66, 34.21, 180.15.

**Tetraethylene glycol dodecanoate disulfide (6)** Dodecanoic acid disulfide, 5, (0.132 g, 0.28 mmol) was dissolved in methylene chloride (15 ml). Dicyclohexylcarbodiimide (DCC) (0.8248 g, 3.9 mmol) and dimethylaminopyridine (DMAP) (0.065 g, 0.53 mmol) were added to the reaction mixture as solids. Tetraethylene glycol (2.7473 g, 14.14 mmol) was then added to the reaction and the reaction was allowed to proceed for 3.5 h. A white precipitate formed and was removed by filtering through celite. The solvent was removed by rotary evaporation, and the reaction taken up in ethyl acetate (50 ml) and washed with water (60 ml). The organic layer was dried with sodium sulfate and the solvent was removed in vacuo. The product was purified by silica chromatography (ethyl acetate:methanol 90:10) to afford a white wax. Yield: 0.352 g (22.8%). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 1.25-1.32 ppm (28 H, m), 1.64-1.7 ppm (8 H, m), 2.38 ppm (4 H, t), 2.72 ppm (4 H, t), 3.6-3.7 (28 H, m), 4.2 (2 H, t). $^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$ 25.11,
28.73, 29.12, 29.34, 29.43, 29.47, 29.69, 29.73, 34.41, 39.38, 61.94, 63.53, 69.46, 70.55, 70.74, 70.76, 70.86, 72.73, 174.09. MS (ESI+) m/z calculated for C_{40}H_{78}O_{12}S_{2} + Na^+ 837.49, found 837.4912.

**Mercaptododecaote tetraethylene glycol (2)** Tetraethylene glycol dodecanoate disulfide (0.052 g, 0.063 mmol) was purged with argon gas and diluted in methylene chloride (5 ml). A 0.4 mmol/ml solution of tributyl phosphine (600 µl, 0.24 mmol) was added and the reaction was allowed to stir for 1.5 h. The solvent was removed by rotary evaporation and the product was purified by silica chromatography (ethyl acetate) to give the product as a white wax. Yield: 16.4 mg (31.5%). $^1$H NMR (300 MHz, CDCl$_3$): δ 1.24-1.32 ppm (14 H, m), 1.70 (4 H, m), 2.2 (2 H, t), 1.9 (2 H, t), 2.36 (2 H, t), 3.44 (2 H, t), 3.6-3.71 (12 H, m), 4.15 (2 H, t). $^{13}$C NMR (300 MHz, CDCl$_3$): δ 25.15, 28.43, 29.02, 29.38, 29.52, 29.71, 33.11, 34.45, 62.02, 63.59, 69.53, 70.60, 70.82, 70.91, 72.75. MS (ESI+) m/z calculated for C$_{20}$H$_{40}$O$_6$S + Na 431.2438, found 431.2429.

### 3.4.2 Synthesis of Amide-linked glycol thiol (3)

**Tosyltetraethylene glycol (7)** Tetraethylene glycol (10.49 g, 54.01 mmol) was dissolved in dry tetrahydrofuran (THF) (20 ml). Pyridine (4.5 ml, 55.18 mmol) was added and the reaction was allowed to proceed for 5 minutes at 0 °C. Recrystallized tosyl chloride (6.79 g, 35.62 mmol) dissolved in dry THF (10 ml) was added to the reaction dropwise and allowed to proceed for 2 hours at room temperature. The reaction was evaporated to dryness, diluted with chloroform (40 ml), and washed with 1 M HCl (40 ml), 1 M NaOH (40 ml), and water (60 ml). The organic layer was dried over sodium sulfate and the solvent was removed by rotary evaporation. The resulting oil was purified by silica
chromatography (ethyl acetate/ hexanes 80:20) to afford the product as a colorless oil. Yield: 4.215 g (34%). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 2.45 ppm (3 H, s), 3.6-3.65 (14 H, m), 7.37 (2 H, d), 7.81 (2 H, d). \(^1^3\)C NMR (300 MHz, CDCl\(_3\)): \(\delta\) 24.3, 61.4, 67.5, 69.1, 70.5, 72.7, 129.6, 130.6, 138.2, 144.3. MS (ESI+) \(m/z\) calculated for C\(_{15}\)H\(_{24}\)O\(_2\)S +H\(_1\) 349.1316, found 349.1313.

**Azidotetraethylene glycol (8)** Tosyltetraethylene glycol, 7, (3.26 g, 9.36 mmol) was refluxed with 95% ethanol (50 ml) and sodium azide (1.4 g, 21.6 mmol) for 19 hours. The solvent was removed by rotary evaporation followed by addition of chloroform (50 ml). This was rinsed with water (75 ml) and the organic layer was dried *in vacuo* to afford the product as a colorless oil. Yield: 1.26 g (61.2%). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 3.4 (2 H, t) 3.6-3.65 (14 H, m). \(^1^3\)C NMR (300 MHz, CDCl\(_3\)): \(\delta\) 50.0, 61.4, 70.0, 70.2, 70.5, 72.7. MS (ESI+) \(m/z\) calculated for C\(_8\)H\(_{17}\)N\(_3\)O\(_4\) +H\(_1\) 220.1325, found 220.1289.

**Aminotetraethylene glycol (9)** Azidotetraethylene glycol, 8, (0.656 g, 3.04 mmol) was dissolved in THF (10 ml) followed by addition of triphenyl phosphine (0.9224 g, 3.52 mmol) as a solid. The reaction was allowed to proceed for 10 h. Deionized water (120 \(\mu\)L, 6.72 mmol) was added to the reaction which proceeded for an additional 13 h. The reaction was diluted by water (40 ml) and rinsed with toluene (50 ml). The water was removed by rotary evaporation to afford the product as a colorless oil. Yield: 0.539 g (91.8%). \(^1\)H NMR (300 MHz; CDCl\(_3\)): \(\delta\) 2.92 (2 H, t) 3.6-3.65 (14 H, m). \(^1^3\)C NMR (300 MHz; CDCl\(_3\)): \(\delta\) 41.39, 61.50, 70.23, 70.38, 70.66, 70.76, 72.59, 73.15. MS (ESI+) \(m/z\) calculated for C\(_8\)H\(_{19}\)NO\(_4\) +H\(_1\) 194.1387, found 194.1382.
12-thioacetatedodecanimidotetraethylene glycol (10) 12-Thioacetatedodecanoic acid, 4, (0.679 g, 2.48 mmol) was dissolved in DMF (20 ml) followed by addition of diisopropylethylamine (875 μl, 5.02 mmol). O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (1.437 g, 3.79 mmol) was added to the reaction as a solid followed by aminotetraethylene glycol, 9, (0.6453 g, 3.33 mmol). The reaction was allowed to proceed for 6 h. The reaction was diluted with dichloromethane (75 ml) and rinsed with water (100 ml). The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed by rotary evaporation. The product was further purified by silica column (ethyl acetate) to afford the product as a colorless oil. Yield: 0.503 g (45.1%). ¹H NMR (300 MHz, CDCl₃): δ 1.3 ppm (14 H, s), 1.60 (4 H, m), 2.18 (2 H, t), 2.3 (3 H, s), 2.86 (2 H, t) 3.43-3.73 (15 H, m). ¹³C NMR (300 MHz, CDCl₃): δ 26.02, 28.99, 29.06, 29.36, 29.60, 29.65, 29.71, 30.88, 36.76, 36.74, 39.29, 61.56, 70.06, 70.46, 70.50, 70.64, 72.65, 72.63, 174.23, 196.30. MS (ESI+) m/z calculated for C₂₂H₄₃O₆NS+Na 472.2703, found 472.2695.

12-mercaptododecanimide tetraethylene glycol (3) 12-

Thioacetatedodecanimidotetraethylene glycol, 10, (0.18 g, 0.356 mmol) was diluted in methanol (10 ml) followed by addition of acetyl chloride (50 μl, 0.857 mmol). The reaction was allowed to reflux for 4 h. The reaction was dried by rotary evaporation, dissolved in chloroform (30 ml), and rinsed with water (30 ml). The solvent was removed in vacuo and the product was further purified by silica chromatography (ethyl acetate) to afford a white wax. Yield: 0.153 g (92.6 %). ¹H NMR (300 MHz, CDCl₃): δ 1.3 ppm (15 H, s), 1.6 (4 H, m), 2.2 (2 H, t), 2.56 (2 H, t) 3.5-3.78 (16 H, m). ¹³C NMR (300 MHz, CDCl₃): δ 24.90, 26.10, 28.62, 29.34, 29.761, 34.30, 36.88, 39.30, 61.81,
70.25, 70.69, 70.91, 72.85. MS (ESI+) m/z calculated for C_{20}H_{41}NO_{5}S +Na 430.2603, found 430.2594.

3.4.3 PDMS Stamp Preparation

*Master Formation.* A virgin silicon wafer (50 mm, Montco Silicon) was cleaned with acetone. AZ 9245 (1-2 mL, Mays Chemical Company, Indianapolis, IN) was applied to the wafer using a Cee 200CB spin/bake system (Brewer Science, Rolla, MO) and an even coating of resist (nominally 4.5 µm) was achieved using a two-cycle spin-coater program (1000 rpm/500 rpm/s/5 s, 3800 rpm/3800 rpm/s/30 s). The wafer was soft baked at 110 °C for 2 min. Photolithography was carried out using a LaserWriter system equipped with a 325 nm laser (Microtech s.r.l., Palermo, Italy). The wafer was developed in 1:2 400K developer (Mays Chemical Company, Indianapolis, IN):deionized water for 2 min. The resulting master was used for stamp formation.

*PDMS Stamp Formation.* Sylgard 182 (Dow Corning, Midland, MI) was mixed 10:1 (resin:hardener) and poured over the patterned silicon master. The polymer was degassed using a vacuum dessicator and cured at 70 °C for 2 h. The final stamp was separated from the master and cut to size.

3.4.4 Patterned Cell Growth

*Patterning SAMs.* Glass coverslips (25 mm, No. 1, VWR, Batavia, IL) were cleaned by oxygen plasma oxidation for 20 min at 100% power. Coverslips were then twice rinsed with water and ethanol, and dried under nitrogen. Deposition of 50 Å titanium followed
by 50 Å, 100 Å, 150 Å, 200 Å, or 250 Å gold onto the glass coverslips was carried out with a PVD 75 electron beam evaporator under vacuum (1 E^-6 - 1 E^-7 Torr).

The stamp was coated with hexadecanethiol (Alfa Aesar, Ward Hill, MA) (10 mM in ethanol) by dropping the solution onto the stamp (5-6 drops) and drying with nitrogen. Slides were then stamped for 10 s. The bare regions of gold were allowed to react with 1 mM (1-mercaptoundec-11-yl)tetra(ethyleneglycol) (1), ester-linked glycol thiol (2), or amide-linked glycol thiol (3) in ethanol for 12-14 h. After soaking, coverslips were twice rinsed with ethanol and dried under nitrogen.

**Cell Culture.** A patterned coverslip (stamped with 10 mM hexadecanethiol and incubated in 1 mM 1, 2, or 3 for 12-14 h) was coated with fibronectin at 20 µg/mL in Dulbecco’s Phosphate Buffered Saline (DPBS, Gibco) at 37 °C for 1 h. Excess protein was removed by rinsing with DPBS (3x) and the coverslip was covered with fresh DPBS. CHO-K1 cells (ATCC, Manassas, VA) were detached using TrypLE Express (Invitrogen), followed by resuspension in Dulbecco’s Modified Eagle Medium (DMEM, low glucose 1X, glutamax, 1 g/L D-glucose, 110 mg/L sodium pyruvate, 10% FBS, 1% penicillin/streptomycin (10,000 units/mL Penicillin G Sodium and 10,000 µg/mL Streptomycin Sulfate in 0.85% saline), Invitrogen), and counted using a hemacytometer (Bright-Line, Hausser Scientific). After rinsing the patterned coverslip with DPBS, approximately 100,000 cells were applied in 1 mL of DMEM. Plated cells were grown at 37 °C and 5% CO_2). Cultures were visualized using live-cell inverted microscopy.

**Analysis of Cells Growing Outside the Pattern.** Cells growing outside the 95 µm circle pattern were counted as either round or spread cells. A cell possessing any projections or
appearing elongated was considered spread, while cells having a round morphology were considered round. Cell density was determined by dividing the number of cells outside the pattern by the background area. Outliers were eliminated using the Grubbs’ test with a critical value of 0.05.

3.4.5 Scanning probe microscopy (SPM)

All SPM images were obtained on a Multimode VIII with Peak Force Quantitative Nanomechanical property mapping (Bruker, Santa Barbra, CA) using a silicon tip on a silicon nitride cantilever with a nominal spring constant of 0.4 N/m (Scanasyst-Air, Bruker Probes, Camarillo, CA). Images of patterned gold substrates were obtained with 512 points per line and 512 lines per image with a frequency of 0.97 Hz. Bare gold substrates were acquired with 1024 points per line and 1024 lines per image with a frequency of 0.488 Hz.
3.5 References


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CHAPTER FOUR

RECYCLING AND REUSING PATTERNED SELF-ASSEMBLED MONOLAYERS FOR CELL CULTURE

4.1 INTRODUCTION

Patterned self-assembled monolayers (SAMs) have been widely utilized for the study of cellular growth and behavior. While microcontact printing is a straightforward method of producing patterned substrates, the process is time consuming and requires the use of many techniques and specialized equipment. Here we present a method by which patterned substrates can be reused up to 15 times, saving both time and valuable resources.

The ability to produce patterned substrates suitable for cell culture has become highly desirable. Control of protein adsorption and cell growth allows for the study of cellular behavior and processes. Patterned substrates have been used to examine cellular confinement, cell migration, cell differentiation, and other important biological phenomena. As a result, these surfaces have the potential to significantly
impact many fields of biology.

Reusable biosensors, DNA microarrays, and microfluidic devices have been developed to provide consistent results over the course of many cycles. Recently, major advances have occurred in these fields with the utilization of self-assembled monolayer chemistry for small molecule and protein detection. A resusable immunosensor for label-free detection of insulin has been produced with monolayers formed from polyethylene glycol (PEG) terminated aklanethiols on gold.\(^9\) This sensor can be reused more than 25 times without loss of function. Biosensors composed of short chain carboxylic acid terminated SAMs have been shown to be reuseable up to 50 times when the thiol monomers forming SAMs are cycled on and off the substrate.\(^{10}\) DNA microarrays have been developed through the use of SAM chemistry as well; allylmercaptan forms the surface to which thiol-terminated short DNA strands are covalently bound and can be released by pulsed plasma and re-formed.\(^{11}\) Each of these devices save time and resources because they are recyclable; however, many of them require release and reformation of the monolayer for reuse.

Here we have established a method by which patterned self-assembled monolayer substrates can be recycled up to fifteen times over the course of many days. We have developed two distinct methods for substrate recycling and reuse, which have the potential to be applied to a broad array of systems. Moreover, we have shown that it is possible to vary the cell lines utilized in cell confinement studies.
4.2 RESULTS AND DISCUSSION

The production of patterned SAMs for cell culture is a multi-step process involving the use of a wide range of techniques. Photolithography is first used to create a patterned master from which a polydimethylsiloxane (PDMS) stamp can be cast. This stamp is then used to microcontact print hexadecanethiol onto an electron beam evaporated gold substrate and bare regions of the gold substrate are backfilled with an amide-linked glycol terminated thiol capable of resisting protein and cellular adhesion.\textsuperscript{12-14} Protein can then be added to the surface to produce a well-defined, stable patterned substrate capable of supporting cell adhesion and growth. The production of a patterned substrate from the start of photolithography, through the process of microcontact printing, and finally to the addition of labeled protein can take anywhere from two days to two weeks or more. We have developed time- and cost-effective methods to create substrates that can be recycled and reused several times without disruption of the integrity of the patterned monolayer. Our system will allow any research laboratory to be able to take advantage of patterned substrates in their studies.

All patterned substrates were formed on glass coverslips coated with 50Å titanium and 150Å gold deposited by electron beam deposition. Microcontact printing of hexadecanethiol was followed by backfilling with the amide-linked glycol thiol, which was shown to resist protein adsorption and cell growth for over five weeks.\textsuperscript{14} The patterned substrates were incubated with a 1:1 mixture of AlexaFluor 647 labeled and unlabeled human plasma fibronectin and CHO-K1 cells were plated onto the substrates.
Recycling and reuse of the patterned substrates was completed in a simple two step cycle (Figure 4.2.1). First, cells previously plated on the substrates were removed by TrypLE Express (a stable trypsin analogue) mediated release or by washing with 1% Triton X-100. Following release, new cells, either CHO-K1 or NIH/3T3, were plated onto the substrates. For each method, cycles of cell removal and cell plating were repeated until the patterned substrate no longer yielded consistent confinement.

In several trials, patterned substrates could be reused up to 15 times over the course of

Figure 4.2.1. Schematic of the production of recyclable patterned substrates.
eleven days when cells were removed by TrypLE Express (Figure 4.2.2). Both CHO-K1 and NIH/3T3 cells were cycled alternately. The cells were confined to 340µm recycle symbols for 4-12 hours between release and reuse of the substrate. Typically, CHO-K1 cells were grown for 4 hours between cycles, while NIH/3T3 cells were grown for 12 hours due to variations in spreading and proliferation rates. No significant defects were found on the substrate until day 11 (cycle 15). A significant defect is observed upon cell spreading and proliferation, rather than attachment of cells possessing a round morphology. Cells exhibiting a round morphology were typically dead and easily rinsed off the substrate with Dulbecco’s Phosphate Buffered Saline (DPBS). In previous studies using the amide-linked glycol, CHO-K1 cells cultured on these substrates and allowed to proliferate without disruption were confined to a pattern for over five weeks. The discrepancy in surface stability is most likely due to the introduction of new cells. In this system, new, healthy cells are repeatedly introduced to the patterned substrate at high concentrations, providing a

![Figure 4.2.2. Fifteen TrypLE Express washing cycles of alternating patterned CHO-K1 and NIH/3T3 cells. Scale bar is 100µm.](image)
greater opportunity for cells to detect and attach at defect sites. The cells can then excrete extracellular proteins onto any defect site creating a larger and more accessible site for subsequent cell attachment and outgrowth. When substrates are used in a single long-term cell culture experiment, greater stability is observed because the monolayer is not disturbed and remodeled as rapidly by the constant addition of high density, healthy cells.

When cells were removed with detergent (Triton X-100), the patterned substrates were stable for up to eight cycles (Figure 4.2.3). As the number of cycles increased, degradation of the protein pattern was observed. The protein pattern was initially

![Figure 4.2.3. Eight detergent washing cycles of CHO-K1 and NIH/3T3 patterned cell growth. Scale bar is 100µm.](image)

![Figure 4.2.4. AlexaFluor 647-labeled fibronectin after washing with 1% Triton X-100 and CHO-K1 or NIH/3T3 patterned cell growth. Scale bar is 100 µm.](image)
very sharp and became increasingly round and ragged along the edges affecting the area of cell growth (Figure 4.2.4). After approximately six cycles, some areas of the substrate became confluent while other areas continued to confine cells to the recycle pattern. This is most likely due to disruption of the monolayer by the detergent. Triton X-100 could associate its hydrophilic head group into the amide-linked glycol thiol SAM, exposing its hydrophobic tail, thus forming defect sites throughout the background. This would allow cells to attach and proliferate outside the pattern.

The TrypLE Express and Triton X-100 recycling methods were tested for SAMs comprised of (1-mercaptoundec-11-yl)tetra(ethyleneglycol), a commercially available ether-linked glycol thiol monomer commonly used in SAM formation. For both methods, only four cycles (in three days) could be completed before the integrity of the patterned monolayer was lost and cell growth was observed outside the pattern (Figure 4.2.5). This suggests that the amide-linked glycol thiol is crucial to the creation of recyclable patterned monolayers.

Each method for cell removal and recycling of the patterned substrate is applicable to different types of studies. The trypsin recycling method is ideal for studies where the main objective is to retain the integrity of the SAM. Since trypsinization does not kill cells, this method would be preferred for research studies that utilize the same cell line for multiple trials. In contrast, the Triton X-100 recycling method is best for experiments involving multiple cell lines. As 1% Triton X-100 rapidly kills cells, the original cell line is no longer viable and can be completely replaced with a subsequent cell line. This protocol can be used in studies where the initial cell line would interfere with the growth of the subsequent cell line.
4.3 CONCLUSIONS

This system of recycling and reusing patterned SAMs provides the ability to rapidly explore cell culture on patterned substrates. Our recycling strategies are compatible with a variety of cell types, as demonstrated through the use of CHO-K1 and NIH/3T3 cell lines. These methods reuse the same patterned substrate providing greater consistency of the pattern and allowing for reproducibility between

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**Figure 4.2.5.** Recycling patterned ether-linked glycol thiol monolayers six times. a) The TrypLE Express mediated release method and b) the Triton X-100 washing method both allow for four full cycles before confinement is lost. Scale bar is 100 µm.
experiments. In addition, our methods drastically reduce the time between trials. A typical patterning experiment takes at least 12-14 hours for substrate preparation. Recycling patterned substrates reduces this time to just five or ten minutes. Here we have shown that recycling patterned substrates will save both time and resources while maintaining well-confined cell studies. Advances in research technology and methodology like those shown here allow the scientific community to perform more research studies using fewer disposable resources.
4.4 MATERIALS AND METHODS

Electron beam deposition was carried out with a PVD 75 electron beam evaporator (Kurt J. Lesker, Clairton, PA). Plasma oxidation was carried out in a Femto standard low pressure plasma system (Diener electronic GmbH+Co. KG, Nagold). Fluorescent and phase contrast images were obtained using a Nikon TE2000-PFS microscope running NIS-Elements imaging software and equipped with a Prior XY stage, EXFO X-Cite series 120PC UV illuminator, Photometrics CoolSNAP monochrome camera, and In Vivo Scientific incubation system.

4.4.1 PDMS Stamp Preparation

Master Formation. A silicon wafer (50 mm, Montco Silicon) was cleaned with acetone. AZ 9245 (1-2 mL, Mays Chemical Company, Indianapolis, IN) was applied to the wafer using a CEE 200CB spin/bake system (Brewer Science, Rolla, MO) and an even coating of resist (nominally 4.5 µm) was achieved using a two-cycle program (1000 rpm/500 rpm/s/5 s, 3800 rpm/3800 rpm/s/30 s). The wafer was soft baked at 110 ºC for 2 min. Photolithography was carried out using a LaserWriter system equipped with a 325 nm laser (Microtech, Palermo, Italy). The wafer was developed in 1:3 400K developer (Mays Chemical Company, Indianapolis, IN):deionized water for 2 min. The resulting master was used for stamp formation.

PDMS Stamp Formation. Sylgard 182 (Dow Corning, Midland, MI) was mixed 10:1 (resin:hardener) and poured over the patterned silicon master. The polymer was degassed using a vacuum dessicator and cured at 70 ºC for 2 h. The final stamp was separated from the master and cut to size.
4.4.2 Patterning SAMs

Glass coverslips (25mm, No. 1, VWR, Batavia, IL) were cleaned by oxygen plasma oxidation for 10 min at 100% power. Coverslips were then twice rinsed with water and ethanol, and dried under nitrogen. Deposition of 50Å titanium followed by 150Å gold onto the glass coverslips was carried out with a PVD 75 electron beam evaporator. The stamp was coated with hexadecanethiol (Alfa Aesar, Ward Hill, MA) (10 mM in ethanol) by dropping the solution onto the stamp (5-6 drops) and drying with nitrogen. Slides were then stamped for 10 s. The bare regions of gold were allowed to react with 1 mM 12-mercaptododecanamide tetraethylene glycol (amide-linked glycol thiol) or 1 mM (1-mercaptoundec-11-yl)tetra(ethyleneglycol) (ether-linked glycol thiol) in ethanol for 12-14 h. After soaking, coverslips were twice rinsed with ethanol and dried under nitrogen.

4.4.3 Patterned Cell Growth

Preparation of Fluorescently Labeled Fibronectin. To 20 µL Human Plasma Fibronectin (1 mg/mL in 100 mM CAPS, 0.15 M NaCl, 1 mM calcium chloride, pH 11.5, Invitrogen, Carlsbad, CA) was added 1 µL of 1 M sodium bicarbonate in sterile water and 2 µL AlexaFluor 647 carboxylic acid, succinimidyl ester (5 mg/mL in DMF, Invitrogen). The reaction was mixed and allowed to proceed at room temperature for 1 h. The reaction was quenched by addition of 3 µL of 1.5 M hydroxylamine in 1 N sodium hydroxide and mixed with 20 µL unlabeled fibronectin.
Cell Culture. A patterned coverslip (stamped with 10 mM hexadecanethiol and incubated in 1 mM amide-linked glycol thiol or 1 mM ether-linked glycol thiol for 12-14 h) in either a Noryl or Teflon cell chamber was coated with fibronectin (prepared as described above) at 20 µg/mL in Dulbecco’s Phosphate Buffered Saline (DPBS) at 37°C for 1 h. Excess protein was removed by rinsing with DPBS (3x) and the coverslip was covered with fresh DPBS. CHO-K1 or NIH/3T3 cells (ATCC, Manassas, VA) were separated using TrypLE Express (Invitrogen), followed by resuspension in Dulbecco’s Modified Eagle Medium for CHO-K1 (DMEM, low glucose 1X, glutamax, 1 g/L D-glucose, 110 mg/L sodium pyruvate, 50 mL FBS, 5 mL penicillin/streptomycin (10,000 units/mL Penicillin G Sodium and 10,000 µg/mL Streptomycin Sulfate in 0.85% saline), Invitrogen) or Dulbecco’s Modified Eagle Medium for NIH/3T3 (DMEM, high glucose 1X, glutamax, 1 g/L D-glucose, 110 mg/L sodium pyruvate, 50 mL NCS, 5 mL penicillin/streptomycin (10,000 units/mL Penicillin G Sodium and 10,000 µg/mL Streptomycin Sulfate in 0.85% saline), Invitrogen), and counted using a hemacytometer (Bright-Line, Hausser Scientific). After rinsing the patterned coverslip with DPBS, approximately 200,000 cells were applied in 1 mL of DMEM. Plated cells were grown at 37°C, 5% CO₂. Live cultures were visualized by inverted microscopy using phase contrast optics and labeled protein was visualized using a Cy5 filter cube (Semrock, Rochester, NY).

Recycling with Detergent. Cells were removed by soaking in 1% Triton X-100 in DPBS for 10-15 min. The substrate was rinsed 6-8x with sterile nanopure water followed by 4x with DPBS. The remaining fluorescent protein pattern was imaged after each
washing (Figure S1). After visualization, substrates were reseeded with CHO-K1 or NIH/3T3 cells at a density of approximately 200,000 cells/dish.

**Recycling with TrypLE Express.** Cells were released from the patterned substrate with TrypLE Express. The slide was rinsed with DPBS then incubated with 1 mL TrypLE Express for 5 min at 37°C. The dish was rinsed 2x with fresh DMEM followed by seeding of CHO-K1 or NIH/3T3 cells at approximately 200,000 cells/dish.
4.5 References


8. Chen, C.; Mrksich, M.; Huang, S.; Whitesides, G.; Ingber, D., Geometric control of


CHAPTER FIVE

SPATIAL CONFINEMENT INSTIGATES
ENVIRONMENTAL DETERMINATION OF
NEURONAL POLARITY

Collaboration with Jad P. Abi-Mansour*

5.1 INTRODUCTION

The localization of specific protein markers, organization of microtubules, and often the extension of a single neurite beyond all others, signify that a neuron has reached maturity and axonal differentiation has taken place. A central question that remains is what causes a particular neurite to distinguish itself from other neurites and differentiate into the axon. While the molecular mechanisms guiding this process have been investigated thoroughly, these studies have not clearly distinguished whether axonal differentiation is predetermined or environmentally determined. Thorough and convincing cases have been argued for both environmental and predetermination based on biochemical

* DMJ designed and performed all the experiments, analyzed the data, and prepared the figures. JPA performed initial experiments to guide pattern design.
For example, work by Dotti and co-workers and Abad-Rodríguez and co-workers led to the determination that plasma membrane ganglioside sialidase (PMGS) is expressed in the neurite which will ultimately become the axon, indicating that axonal differentiation is a predetermined process (Figure 5.1.1). This goes directly against literature precedence, which demonstrated that neuronal polarization was environmentally determined based on immunohistochemical and ultrastructural studies. Pennypacker, et al. argued differentiation was not a predetermined process because both microtubule associated protein 2 (MAP2) and phosphorylated high neurofilament subunit (NF-H) are co-expressed early in development and are not segregated until later in maturity (Figure 5.1.1). To clearly distinguish between these two mechanisms, we have taken a chemical biology approach using self-assembled monolayers to generate well-defined environments in order to study differentiation.

Previously, Stenger et al. produced a patterned substrate with “adhesive” and “non-

![Figure 5.1.1. Depiction of axonal and dendritic markers in neuronal polarization. PMGS is localized in the axon prior to axonal differentiation whereas NF-H and MAP2 are co-localized until polarization and is then compartmentalized. NF-H is localized in the axon and MAP2 is located in the dendrites.](image)
adhesive” regions consisting of a single 190 µm solid line along with three broken lines connected to a central site for cell body adhesion. In their study, hippocampal neurons were predominantly found on the pattern and tended to develop a single process that stained positive for NF-H, an axonal marker. While this study provided evidence that it might be possible to control axonal growth environmentally, lack of precise control over the substrates prevented detailed investigation of axon/dendrite differentiation.

Here, we utilized self-assembled monolayer (SAM) chemistry in conjunction with microcontact printing to generate a series of starburst patterns consisting entirely of solid laminin-coated lines in which we systematically vary the path length to assess whether axon/dendrite differentiation is predetermined or environmentally determined. The use of well-defined SAMs prepared from hexadecanethiol and an amide-linked glycol-terminated alkanethiol on gold substrates allows for precise control over the surface chemistry. Patterned substrates, which contain defined laminin regions that potentially mimic the spatial confinement encountered by neurons during development in vivo, can be generated by taking advantage of the fact that glycol-terminated regions of the substrate resist non-specific protein adhesion and hexadecanethiol regions promote non-specific protein adhesion. Using this method, we show that axonal differentiation in E18 mouse hippocampal neurons under steady-state conditions is environmentally determined based on the length a neurite is allowed to grow. Moreover, systematic variation of the pattern has allowed us to examine the critical length a neurite must be allowed to extend past all others to induce axonal differentiation.
5.2 Results and Discussion

Patterned SAMs were created by microcontact printing hexadecanethiol onto electron-beam deposited gold-coated glass coverslips (50Å Ti/150Å Au) using a polydimethylsiloxane (PDMS) elastomer stamp (Figure 5.2.1). Bare regions of the gold

![Figure 5.2.1. Method for fabrication of patterned substrates for neuronal growth. Hexadecanethiol is microcontact printed onto the substrate and the bare regions are backfilled with an amide-linked glycol-terminated alkanethiol. Subsequently, laminin is adsorbed onto the hexadecanethiol monolayer, while the glycol region prevents protein adsorption and allows for neuronal confinement. Scale bar is 50 µm.](image-url)
substrate were backfilled with an amide-linked glycol-terminated alkanethiol. The background glycol-terminated alkanethiol monolayer is well established to resist non-specific protein adsorption and is capable of confining cells to well-defined patterns. Laminin, a common extracellular matrix protein found in vivo and used in neuronal culture because it promotes neurite outgrowth in a non-directive manner, was non-specifically adsorbed to the hexadecanethiol to produce a series of laminin-coated starburst patterns.

The dimensions of the pattern were tuned to properly accommodate hippocampal neurons. We determined that when the center of the starburst was 10 µm and paths were 2 µm wide, the cell bodies of E18 mouse hippocampal neurons attached to the center of the pattern and neurites grew out along the paths. When larger starburst centers were used, we observed multiple cell body attachment and the differentiation of individual neurons could not be resolved. Larger path widths resulted in somal attachment along the paths of the starburst and an elongated morphology, but the cell remained confined within a single path.

Neurons were allowed to reach Stage 3, full differentiation, by culturing for four days in vitro. Banker and co-workers performed ultrastructural studies that showed differences in ribosome concentration in the axon and dendrites in cells devoid of intercellular contacts, verifying that differentiation occurs without natural communication pathways in place. These studies suggest that neurons without synaptic connections differentiate similarly to neurons in complex networks. After four days in culture, cells were saponin extracted, fixed, and permeabilized. Axons were immunostained for microtubule-associated protein tau, which following saponin extraction remains bound to
microtubules and localized in the axon$^{2,22}$ The entire neuron was visualized by staining for actin filaments using fluorescently labeled phalloidin and the laminin pattern was visualized using AlexaFluor 350 labeled anti-laminin antibody (Figure 5.2.2)$^{23}$

A series of starburst patterns with one path longer than the others was created to test the hypothesis that axon/dendrite differentiation was environmentally determined with the critical determinate being the length a neurite was allowed to grow. To test this

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**Figure 5.2.2.** Immunohistochemical analysis of E18 mouse hippocampal neurons confined to the starburst pattern. Representative images of neurons growing on the starburst pattern are shown where a single axon is growing along the long path (A-C) and on the short path (D-F).

The whole neuron is visualized by staining with AlexaFluor 594 phalloidin (A, D), microtubule-associated protein tau is visualized by immunostaining with anti-tau antibody (B, E), and the laminin starburst pattern is visualized by immunostaining with AlexaFluor 350-conjugated anti-laminin antibody (C, F). White arrowheads indicate the neurite that is positive for tau. Scale bar is 25 µm.
hypothesis, we examined starburst patterns consisting of twelve 2 µm wide paths. Eleven of these paths were designed to support dendritic processes and were 20 µm in length. One of these paths was designed to support the axon and this path length was varied from 40 µm to 160 µm in 20 µm increments. If axon/dendrite differentiation were a predetermined process, meaning a single neurite was destined to become the axon, we would expect to see a statistical distribution of axons on all of the paths in the starburst pattern. This would result in the axon being formed on the long path of the starburst pattern approximately 8% of the time. However, we observe that even for the pattern containing eleven 20 µm paths and one 40 µm path, the axon forms on the long path 58% of the time. This is far greater than what would be predicted statistically and, in and of itself, suggests that permissible neurite path length is a critical factor in axon/dendrite differentiation.

What is even more striking than the observation that a difference of only 20 µm in length can greatly influence differentiation is that axonal differentiation depends linearly on length (Figure 5.2.3, a & b) with our 160 µm path length approaching near quantitative axon formation on the long path. When the path length is plotted versus the percent occurrence of the axon on the long path, the data can be fit to a straight line with an $R^2$-value of 0.87 and a significant p-value of 0.0020. This linear correlation clearly shows that axon/dendrite differentiation is environmentally dependent with the critical factor being the length a neurite is allowed to grow. In our simplified in vitro model, this length is defined by the laminin starburst pattern, however in vivo this length is likely defined by the growth cone’s interaction with a myriad of attractive and repulsive guidance cues.24-26
In order to probe the ability of a neurite to discriminate between longer and shorter paths, we developed a second series of patterns in which we varied the length of all the paths in the starburst pattern, but kept the distance between the single long path and the short paths fixed at 20 μm. Recall that in our initial series of starburst patterns, a 20 μm difference in length between the long path and the short paths resulted in the axon being formed on the long path 58% of the time. Here we hypothesized that as we increased the

![Figure 5.2.3](image)

**Figure 5.2.3.** Length dependence of neuronal polarization. Neurons were grown on starburst patterns consisting of 20 μm short paths and a single long path of varying lengths (A). The percentage of neurons with a single axon growing on the long path increases linearly as the path length is increased (B). Neurons were also grown on starburst patterns containing one path 20 μm longer than the short paths (C). The percentage of neurons with a single axon growing on the long path decreases linearly as the path length is increased (D). The sample size associated with each data set is shown in parentheses. Scale bar is 25 μm.
overall length of the short paths, we would approach a statistical distribution. This is observed with the data fitting to a straight line with an \( R^2 \)-value of 0.98 (Figure 5.2.3, c & d). Interestingly, the largest starburst pattern with paths of 80 \( \mu \)m and 100 \( \mu \)m did not support neuronal attachment or axon/dendrite differentiation of healthy neurons. This observation further highlights the importance of an environment conducive to proper axon/dendrite differentiation and suggests \textit{in vivo} repulsive guidance cues must inhibit neurites, which ultimately become dendrites, thus restricting their growth. In addition, neuronal health is often enhanced when the formation of synaptic connections is allowed. All of our starburst patterns prevent these connections from being made in order to observe neuronal behavior in the absence of cell-cell interactions. The lack of healthy neurons on the largest starburst pattern indicates that these cells undergo a greater amount of stress and are unable to adapt.

5.3 CONCLUSIONS

We have demonstrated the environmental dependence of axon/dendrite differentiation by physically altering the environment of E18 mouse hippocampal neurons. Through the use of well-defined patterned substrates produced using self-assembled monolayer chemistry, we have observed a linear relationship between the length a developing neurite can travel and its propensity to differentiate into an axon. Moreover, we have shown that when all neurites can grow long distances, it is difficult to predict which neurite will become the axon and the resulting neurons are less healthy. Our findings are significant because they demonstrate the critical role of environment on the growth and proliferation of a neuron. This study provides valuable insights into neuronal behavior.
both in vivo and in vitro. It is clear from these studies that neurons confined to a specific growth pattern adjust to their environment and differentiate accordingly. Our approach is complementary to traditional biological and biochemical studies and has allowed us to gain insights into a problem that is intractable using classical methods.
5.4 MATERIALS AND METHODS

Gold-coated coverslips, master fabrication, microcontact printing with hexadecanethiol, and backfilling with amide-linked glycol-terminated alkanethiol were performed as previously described.\textsuperscript{18,21}

CD-1 timed pregnancy mice were sacrificed and E18 hippocampus pairs were dissected (Charles River Laboratories, Wilmington, MA). E18 mouse hippocampi were stored in Hibernate E (BrainBits, LLC, Springfield, IL) and later dissociated using a GentleMACS system in conjunction with the Neural Tissue Dissociation Kit (P) and Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA). Mouse Laminin I (Trevigen, Gaithersburg, MD) was deposited onto patterned substrates at 24 µg/mL for 1 h prior to plating. Neurons were seeded onto patterned substrates in Neurobasal medium supplemented with 2% B27 supplement, 1% Glutamax, 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). Cells were stored at 37 °C, 5% CO\textsubscript{2}. After four days in vitro, cultures were subjected to 0.02% saponin extraction as previously described followed by fixation in a 3.7% paraformaldehyde/PHEM buffer and permeabilization with 0.1% Triton X-100.\textsuperscript{22} Substrates were blocked in 10% normal goat serum (Invitrogen, Carlsbad, CA) and neurons were stained using anti-tau, clone 5E2 antibody (Millipore, Temecula, CA), AlexaFluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) AlexaFluor 594 phalloidin (Invitrogen, Carlsbad, CA), and anti-laminin antibody (Millipore, Temecula, CA) which was conjugated to AlexaFluor 350 carboxylic acid, succinimidyl ester (Invitrogen, Carlsbad, CA). Coverslips were subsequently mounted using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). Fluorescent images were obtained using a
Nikon TE2000-PFS microscope running NIS-Elements imaging software and equipped with an EXFO X-Cite UV illuminator and Photometics CoolSNAP camera.

Images were analyzed and individual neurons were reported as containing a single axon, multiple axons, or no axon. Single axon, multiple axons and no axon classifications were similar for both patterned and non-patterned hippocampal neurons at day 4 (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Non-Patterned Neurons</th>
<th>Patterned Neurons</th>
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<tbody>
<tr>
<td>One Axon</td>
<td>71.79%</td>
<td>85.24%</td>
</tr>
<tr>
<td>Multiple Axons</td>
<td>17.95%</td>
<td>10.00%</td>
</tr>
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</tr>
<tr>
<td>Sample Size</td>
<td>39</td>
<td>210</td>
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Table 5.4.1. Neurons were characterized according to the number of neurites staining positive for tau. Both patterned and non-patterned neurons were saponin extracted, fixed and stained at day 4.

Those with a single axon were classified as growing on either the long path or short path.

Data was collected and analyzed for each starburst pattern. Graphs were plotted and $R^2$ and p-values were calculated using KaleidaGraph 3.6 (Synergy Software) and Prism 5 (GraphPad Software, Inc.).
5.5 References


CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

The ability to use a combination of photolithography, materials science, organic chemistry, and biological chemistry has led to the discovery of new, stable materials for the study of biological processes and cellular confinement. Initial studies involving patterned trichlorosilane self-assembled monolayers (SAMs) on glass paved the way for the creation of more stable functionalized substrates, specifically the amide-linked glycol-terminated thiol on gold system, which is stable for more than five weeks in culture. In addition, we developed a patterned substrate system that could be reused multiple times, allowing researchers who cannot afford the costly equipment associated with the fabrication of gold substrates, patterned masters, and patterned SAMs to carry
out cell confinement studies. Through our own use of these stable, patterned substrates, we have gained insights into the process of axonal differentiation.

In chapter two, *Direct Printing of Trichlorosilanes on Glass for Selective Protein Adsorption and Cell Growth*, we introduced a method for the creation of patterned glass substrates using trichlorosilane self-assembled monolayer chemistry. The pattern was formed by microcontact printing octadecyltrichlorosilane onto a glass coverslip and backfilling with glycol-terminated trichlorosilane. This resulted in a patterned substrate for protein and cell confinement. Chinese hamster ovary (CHO-K1) cells were well-confined to the fibronectin-coated circles pattern. While this system allows for the easy and fast functionalization of glass substrates, the trichlorosilane monomers are highly reactive to moisture in the air and are therefore difficult to work with. In cases where a glass substrate is the only viable option, this system is a good choice for producing a patterned surface.

The desire to develop a patterned substrate capable of long-term stability led us to our work in chapter three, *Increased Stability of Glycol-Terminated Self-Assembled Monolayers for Long-Term Patterned Cell Culture*. Alkanethiols on gold have been a well-established, meticulously studied SAM system for the past thirty years;\(^1\)\(^{-10}\) our goal was to produce a system that could outlast previous gold/thiol approaches and maintain stability under cell culture conditions. With the incorporation of an amide-linkage between the alkane chain and glycol termination, we were able to extend cell confinement studies to over five weeks in culture. This surpassed previous attempts, such as the mannitol-terminated and D+L gulitol systems,\(^11\),\(^12\) by more than 10 days. Further, through the use of quantitative nanomechanical mapping (QNM) and scanning
probe microscopy (SPM), we discovered the mechanism for monolayer degradation is not monomer mixing or adlayer blooming, rather, it is thiol oxidation. In addition, we found that nanotopology plays a large role in the stability of the monolayer, so much so that an increase of 100 Å gold, from 100 Å gold to 200 Å gold, decreases the stability of the monolayer by three weeks.

The advantage of increased stability allowed for the development of recyclable patterned substrates as evidenced in chapter four, *Recycling and Reusing Patterned Self-Assembled Monolayers for Cell Culture*. Using two different approaches, were able to reuse patterned substrates up to 11 times over the course of 15 days in culture. The traditional glycol-terminated SAM system, consisting of an ether-linkage, was only stable for four uses in three days. The two methods employed in recycling patterned SAMs were TrypLE Express-mediated cell detachment and removal and detergent-assisted cell death and removal. Each strategy has its own advantages: TrypLE Express (a trypsin analog) allows for the gentle removal of cells for repetitive studies, whereas detergent-assisted cell removal is useful in studies involving multiple cells types that may interfere with or be detrimental to one another. Overall, this work allows a wide range of researchers to utilize patterned substrates for biological studies.

Our focus for biological studies utilizing two-dimensional confinement has centered on neuronal development, specifically axonal differentiation. As discussed in chapter five, *Spatial Confinement Instigates Environmental Determination of Neuronal Polarity*, we have created starburst patterned substrates that confine neurons and allow a single neurite to grow along a defined length. By forcing which neurite can grow longer than the rest, we were able to study whether neuronal polarization is predetermined or environmentally
determined. If the long neurite becomes the axon in a significant number of cases, more than ~8% of the time, then we would find that axonal differentiation is environmentally determined. In fact, we found that when a neurite is allowed to grow just 20 µm past all others, it became the axon 58% of the time, signifying environmental determination as the key component in axonal differentiation. In addition, we were able to ask interesting questions about path length dependence, specifically whether a neuron is capable of distinguishing which path is longest when all paths are greater than 40 µm. We found that the ability of a neuron to sense which path is longest in these cases diminishes significantly as the path length increases.

6.2 **Future Directions**

These initial studies have given rise to several possibilities for future directions. Two of which are demonstrated in Appendix One, *Further Studies of Axonal Differentiation*. First, we were interested in whether the overexpression of plasma membrane ganglioside sialidase (PMGS) would alter the findings of our initial axonal differentiation studies. If you recall, PMGS was found to localize in the single neurite destined to become the axon, suggesting that polarization is a predetermined process. We have begun studies to test whether this innate behavior is maintained in E18 mouse hippocampal neurons transfected with PMGS-mCherry and grown on starburst patterned substrates. As another extension of our axonal differentiation work, we aim to study starburst patterned substrates comprised of N-cadherin rather than laminin. Laminin is a basement membrane protein we commonly use to aid in neuronal attachment and neurite outgrowth because it promotes neurite extension nondirectionally, allowing cells to develop
without attraction or repulsion to any particular part of the pattern. N-cadherin is responsible for cell-cell attachment and interactions. Therefore, we wanted to study how or whether this would coincide with our previous studies. In addition, it would be interesting to produce substrates composed of fibronectin and collagen patterns to observe variations in axonal differentiation for two-dimensionally confined hippocampal neurons. Another variation of this study would include the incorporation of guidance cues into the starburst pattern. Negative cues would likely not be interesting since the cell would not be able to attach or grow. However, positive guidance cues could force either more rapid differentiation or possibly multiple axons within a single cell if the cue forces upregulation of genes responsible for multiple axon differentiation, such as CRMP-2.

Another future objective, which is currently underway in our group, is to expand the starburst pattern to create neuronal networks. Initial studies did not allow cellular interactions or connections to occur. In neuronal networks, information feedback

![Figure 6.2.1](image)

**Figure 6.2.1.** Example of a starburst patterned network that would force a single axon to grow along a short path (A) or would allow each axon to grow down a long path (B).
mechanisms may allow for a variety of outcomes. For example, a network consisting of three starburst patterns with only two connection points would likely result in a single neuron being forced to extend its axon along a short path (Figure 6.2.1). However, if three connections were present, this would likely not happen. As the complexity of the network increases, we would learn more about neuronal communication, specifically when a patterned network is used in conjunction with a microelectrode array (MEA). In this case, one could excite a single neuron and observe its interactions with other cells in real time. An alternative would be to utilize a calcium-sensing dye to visualize communication routes through live-cell imaging.

Through our axonal differentiation studies, we have developed a collaboration with Axion Biosystems, an Atlanta-based biotechnology company, to study defined neuronal networks on biologically compatible MEAs. This work is similar to the above-mentioned project, however, initial studies involve a network pattern consisting of multiple sites for somal adhesion connected to one another. We will be patterning directly onto prepared MEAs and functionalizing the substrate in the same fashion as in chapter five. This collaboration will provide a basis for later work involving our starburst pattern networks on MEAs.

An additional method we can employ to study axonal differentiation and neuronal behavior is grayscale photolithography. This technique will facilitate the fabrication of three-dimensional and quasi-three dimensional substrates. Currently, we have developed a working method for the analysis of neuronal growth on quasi-three dimensional wave patterned substrates, involving the use of gold-coated, patterned Norland 68 optical adhesive and scanning electron microscopy (SEM) analysis. A wide variety of substrates
and fabrication methods were tested, as evidenced in Appendix Two, *Fabrication of Quasi Three-Dimensional Wave Patterns to Study Neuronal Behavior*, before arriving at this final substrate preparation.

Ideally, our long-term stability studies, two-dimensional cell confinement work, and three-dimensional substrate preparation will be united to develop patterned three-dimensional substrates for the study of biological processes. This type of substrate could be developed to mimic *in vivo* conditions more closely than any two-dimensional substrate and would provide valuable insights into neuronal behavior *in vivo*. 
6.3 References


APPENDIX ONE

FURTHER STUDIES OF AXONAL DIFFERENTIATION

A1.1 OVERVIEW

In chapter five, we confined hippocampal neurons to a two-dimensional starburst pattern in order to examine whether axonal differentiation was preprogrammed or environmentally determined. We utilized laminin on the substrate to provide an adhesive yet non-directional surface for neurons to attach and grow. Our data showed us that polarization is an environmentally determined event dependent on the distance a neurite is allowed to grow. As an extension of our previous work on the study of axonal differentiation, we have chosen two methods that will shed further light on the mechanism of neuronal polarization. The first method involves the creation of a neural cadherin (N-cadherin) pattern rather than a laminin one. We are interested in whether N-
cadherin, a protein responsible for cell-cell adhesion, will elicit a different response from a neuron than what was observed for laminin. The second method incorporates overexpression of plasma membrane ganglioside sialidase (PMGS) in neurons confined to a starburst pattern. In non-patterned neurons, overexpression of PMGS led to rapid axonal differentiation. It would be interesting to determine whether this would lead to data that points toward axonal differentiation as a predetermined event or if we would still find it to be environmentally determined. Both of these methods have produced promising preliminary data and could provide essential information about the mechanism of axonal differentiation.

A1.2 N-CADHERIN STUDIES

Neural cadherin is an extracellular matrix (ECM) protein responsible for calcium dependent cell-cell adhesion and interactions. This specific cadherin is found primarily within neuronal networks, however, there are many other types of cadherins responsible for various cellular connections within the body. Tanaka et al. found that N-cadherin is modified by synaptic activity, more specifically, it undergoes conformational changes, dimerizes, becomes more protease resistant, and redistributes itself upon synaptic stimulation. Bixby et al. discovered that N-cadherin promotes neurite outgrowth similar to that observed with laminin; however, the mechanism inducing neuronal growth is likely different. We focused on N-cadherin as a target for our patterned neuronal studies because it would provide a more adhesive substrate for neuronal growth than laminin. Also, neurons respond differently to different ECM proteins and that may lead to changes in axonal differentiation.
The first difference noticed between E18 mouse hippocampal neurons grown on N-cadherin rather than laminin is that the cells appear healthier and a higher percentage of plated cells adhere to the pattern and produce neurites. Within 24 hours, neurons have nearly filled the starburst patterns. This is likely due to the inherent nature of the protein itself since it is an adhesive ECM protein.

This study appeared to be very promising from the beginning due to the impressive neuronal growth as well as the health of the neurons confined to the pattern. Often neurons that are not allowed to make connections become unhealthy after several days in culture; however, neurons cultured on N-cadherin continued to survive for about a week. Additionally, the neurons produced a greater number of projections on N-cadherin than on laminin. While the cells thrived, immunostaining proved to be our biggest issue. We received the N-cadherin protein from the Leckband group at the University of Illinois Urbana-Champaign. They had expressed the recombinant protein with an FC-tag for purification purposes. Initially, we thought this would be to our advantage because we could use a fluorophore-conjugated protein A to visualize the patterned protein. However, protein A did not interact strongly with the FC-tag and we were unable to see the starburst pattern. Next we aimed to image the neuron, tau protein, and surface N-cadherin by utilizing the same protocol as in chapter five, except with an anti-N-cadherin antibody. This failed because of the strong interaction between the goat anti-mouse IgG whole antibody and the FC-tagged N-cadherin on the surface (Figure A1.2.1). We could not visualize the tau protein bound to microtubules within the cell and therefore we could not determine which neurite was the axon. In another trial, each antibody was conjugated to a different fluorophore prior to immunostaining; however, this afforded no pattern
The final protocol we attempted involved the use of a secondary antibody for the anti-tau primary antibody that consisted of the IgG F(ab')\textsubscript{2} fragment. The F(ab')\textsubscript{2} secondary is not raised against primary antibodies with the FC portion and therefore it should not interact with the tagged N-cadherin. Despite this, the patterned N-cadherin is still visualized in the green channel, which is reserved for imaging tau (Figure A1.2.2). Therefore, we have been unable to image tau, to determine the position of the axon, and N-cadherin in different channels. In the future, it would be best to use recombinant N-cadherin without an FC purification tag, which would allow us to use the immunostaining

**Figure A1.2.1.** E18 mouse hippocampal neuron confined to an N-cadherin starburst pattern. Immunostaining was carried out for actin (A), tau (B), and N-cadherin (C). The AlexaFluor 488 goat anti-mouse IgG (H+L) reacted strongly with N-cadherin on the surface. Scale bar is 20 µm.

**Figure A1.2.2.** E18 mouse hippocampal neuron confined to an N-cadherin starburst pattern. Immunostaining was carried out for actin (A), tau (B), and N-cadherin (C). The AlexaFluor 488 goat anti-mouse F(ab’)\textsubscript{2} fragment reacted strongly with N-cadherin on the surface. Scale bar is 20 µm.
protocol in chapter five to determine which path contains the axon.

A1.3 OVEREXPRESSION OF PMGS STUDIES

Previously it was determined that PMGS was localized in a single neurite destined to become the axon.\(^1,8\) Our studies demonstrated that differentiation is an environmentally determined process. The combination of a PMGS study with our axonal differentiation studies could provide further evidence for the mechanism of neuronal polarization. Here we intend to combine overexpression of PMGS-mCherry in E18 mouse hippocampal neurons plated onto a starburst patterned substrate with live-cell epi-fluorescence imaging. We will visualize PMGS localization and movement in real time to determine whether axonal differentiation is preprogrammed or environmentally determined in these confined neurons. In non-patterned neurons, overexpression of PMGS results in polarization within 24 hours. This is much more rapid than in naturally developing cells, which would differentiate at Stage 3, 2-4 days \textit{in vitro}. Therefore, transfection of PMGS-mCherry could result in very different data than that observed in chapter five.

Traditional cloning techniques were carried out to develop the PMGS-mCherry plasmid. Purification of this DNA from Top10F’ cells resulted in DNA with very low protein contamination, making it suitable for neuronal transfection. Several different parameters for nucleofection with the Lonza Amaxa Nucleofector were attempted; however, survival was extremely low and viable neurons were not found after 24 hours. Given the correct transfection conditions, this study would provide new insights into axonal differentiation and would be a valuable study of patterned cell growth in conjunction with the manipulation of gene expression.
A1.4 MATERIALS AND METHODS

A1.4.1 N-Cadherin Studies

Preparation of patterned substrates and procedures for dissection and dissociation of mouse hippocampal tissue were previously described in chapter five, *Spatial Confinement Instigates Environmental Determination of Neuronal Polarity*. However, N-Cadherin was used in place of Mouse Laminin I. N-Cadherin was expressed and purified with an FC-tag by the Leckband group at the University of Illinois Urbana-Champaign. The protein was deposited onto patterned coverslips at 5 μg/mL HEPES Buffer (pH 7.5, 20mM HEPES, 50mM NaCl, 2mM CaCl₂ in nanopure water) for two hours prior to plating. After four days *in vitro*, cultures were subjected to 0.02% saponin extraction as previously described followed by fixation in a 3.7% paraformaldehyde/PHEM buffer and permeabilization with 0.1% Triton X-100. Substrates were blocked in 10% normal goat serum (Invitrogen, Carlsbad, CA) and neurons were initially stained using anti-tau, clone 5E2 antibody (Millipore, Temecula, CA), AlexaFluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA), AlexaFluor 594 phalloidin (Invitrogen, Carlsbad, CA), and anti-N-Cadherin antibody (Sigma-Aldrich, St. Louis, MO) which was conjugated to AlexaFluor 350 carboxylic acid, succinimidyl ester (Invitrogen, Carlsbad, CA). Several methods of immunostaining were tested due to the presence of the FC-tag on N-Cadherin and are outlined in Table A1.4.1. Coverslips were subsequently mounted using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). Fluorescent images were obtained using a Nikon TE2000-PFS microscope running NIS-
Elements imaging software and equipped with an EXFO X-Cite UV illuminator and Photometrics CoolSNAP camera.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Actin</th>
<th>1° Tau</th>
<th>2° Tau</th>
<th>N-Cadherin</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AlexaFluor 594-phalloidin (Invitrogen)</td>
<td>anti-tau, clone 5E2 (Millipore)</td>
<td>DyLight 488 sheep anti-mouse IgG (H+L) (VWR)</td>
<td>DyLight 405 conjugated Protein A (VWR)</td>
<td>No pattern observed</td>
</tr>
<tr>
<td>2</td>
<td>AlexaFluor 594-phalloidin (Invitrogen)</td>
<td>anti-tau, clone 5E2 (Millipore)</td>
<td>AlexaFluor 488 goat anti-mouse IgG (H+L) (Invitrogen)</td>
<td>AlexaFluor 350-conjugated anti-N-Cadherin (Sigma-Aldrich)</td>
<td>goat anti-mouse IgG interacted too strongly with N-Cad</td>
</tr>
<tr>
<td>3</td>
<td>AlexaFluor 594-phalloidin (Invitrogen)</td>
<td>AlexaFluor 488 conjugated anti-tau, clone 5E2 (Millipore)</td>
<td>AlexaFluor 350-conjugated anti-N-Cadherin (Sigma-Aldrich)</td>
<td>No tau visible, very difficult to see starburst pattern</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AlexaFluor 594-phalloidin (Invitrogen)</td>
<td>anti-tau, clone 5E2 (Millipore)</td>
<td>AlexaFluor 488 goat anti-mouse F(ab’)_2 fragment (Invitrogen)</td>
<td>AlexaFluor 350 goat anti-mouse IgG (H+L) (Invitrogen)</td>
<td>High reactivity with goat anti-mouse F(ab’)_2 fragment, could not see tau staining</td>
</tr>
</tbody>
</table>

Table A1.4.1. Immunostaining parameters for N-Cadherin studies.

A1.4.2 Overexpression of PMGS Studies

Strains and Plasmids

Subcloning was conducted using the Top10F™ E. coli strain (Invitrogen, Carlsbad, CA), starting with the pmCherry-C1 vector (Clontech, Mountain View, CA) and PMGS in the pCR4-TOPO vector (Thermo Scientific, Waltham, MA).

Cloning

PMGS image clone (ID# 40126211) from mouse cDNA was obtained from Open Biosystems (Thermo Scientific). The gene was cloned using traditional cloning techniques into the XhoI and EcoRI sites in the pmCherry-C1 vector. The m-Cherry tag
was situated at the N-terminus of the PMGS gene. Cloning primers can be found in Table A1.4.2. Cloned sequences were verified by automated DNA sequencing (Big Dye v3.1; Applied Biosystems).

<table>
<thead>
<tr>
<th>Cloning Primer</th>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMJ-001</td>
<td>Forward, XhoI site</td>
<td>GCC TCT CGA GCT GAA GCC ATG GAG G</td>
</tr>
<tr>
<td>DMJ-002</td>
<td>Reverse, EcoRI site</td>
<td>CTC TAA GAA TTC GAG ACC TCT GTT AC</td>
</tr>
</tbody>
</table>

*Table A1.4.2.* Cloning primers for the insertion of PMGS cDNA into the pmCherry-C1 vector.

**E18 Mouse Hippocampal Transfection**

CD-1 timed pregnancy mice were sacrificed and E18 hippocampus pairs were dissected (Charles River Laboratories, Wilmington, MA). E18 mouse hippocampi were stored in Hibernate E (BrainBits, LLC, Springfield, IL) until dissociation. Mouse Laminin I (Trevigen, Gaithersburg, MD) was deposited onto glass substrates at 12 µg/mL Dulbecco’s Phosphate Buffered Saline (DPBS) overnight prior to plating. Hippocampi were dissociated using a GentleMACS system in conjunction with the Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, Auburn, CA). Neurons were transfected using the Lonza Amaxa Nucleofector following their optimized protocol for primary mouse hippocampal and cortical neurons (Lonza, Cologne AG, Cologne). Nucleofection buffer used was made in-house by combining 80 µL Solution I (363 mM ATP-Mg salt, 590 mM magnesium chloride hexahydrate in sterile water) with 4 mL Solution II (88 mM potassium dihydrogen phosphonate, 14 mM sodium bicarbonate, 2 mM glucose, pH 7.4 in sterile water) prior to use. Transfected neurons were seeded onto glass substrates in...
Neurobasal medium supplemented with 2% B27 supplement, 1% Glutamax, 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured at 37 °C, 5% CO₂.
A1.5 REFERENCES


A2.1 OVERVIEW

Neurons are exposed to a myriad of guidance cues, both physical and chemical, during development.¹⁻⁸ The axon and dendrites are guided to the appropriate location in the organism by attractive, repulsive, and permissive guidance cues. In addition to the chemical guidance cues that come to mind first, there are also microtopographical variations in vivo that are responsible for the regulation of neuronal growth as well as guiding the direction of neurite outgrowth.⁹ Here we aim to study how microtopography may influence growth rates as well as differentiation by employing quasi three-dimensional wave substrates.
A2.2 INTRODUCTION

Several groups have succeeded in studying a variety of cellular properties using topologically defined substrates. Chen and co-workers are well known for the micropost studies that they have pioneered to look at the behavior of cells in response to changes in mechanical force.\textsuperscript{10,11} They have also used these substrates to characterize the forces a cell exerts on its surroundings by measuring displacement of the posts.\textsuperscript{12,13} Kapoor et al. have focused on studying how microgrooves affect cell attachment and proliferation and gene expression, showing that cells prefer the grow along the grain of the pattern rather than against it.\textsuperscript{14} Another method for investigating cellular response to changes in microtopography is achieved through the fabrication of nanogrooves.\textsuperscript{15} Ferrari et al. studied PC12 cell differentiation, dedifferentiation, and alignment on nanogratings in both the absence and presence of nerve growth factor (NGF) and found that cells preferentially aligned with the nanogrooves.

The work that led us to develop methods for generating smooth, continuous 3D substrates was that of Li and Folch.\textsuperscript{16} They produced three-dimensional ‘step’ substrates from PDMS that consisted of varying step heights ranging from 2.5 to 69 \( \mu \)m. Neurites were unable to climb up walls of 22 \( \mu \)m or greater. They attributed this to the size of the growth cone and presumed that because the growth cone cannot sense an area larger than approximately 15 \( \mu \)m, it is not aware of the top of the step and will only grow along the step rather than over it.
While many groups have studied the effects of microtopography on cell growth, they have not employed grayscale lithography. As a result, the substrates utilized are composed of right angles, which are unlikely to be present *in vivo*. Our approach is unique in that we are attempting to more closely mimic the conditions a neurite may face during guidance by creating smooth, rolling waves for cell growth, as in Figure A2.2.1.

In addition, the use of a continuous, three-dimensional substrate will allow us to ascertain the degree to which neurons are able to ‘climb’ a hill. Through these studies, we will further our knowledge of neuronal behavior, potentially indicating how well neurons can sense their surroundings *in vivo*.

**A2.3 Preliminary Results and Discussion**

Some of the properties we are interested in studying are the vertical distance a neurite is able to overcome as well as the maximum angle of growth from normal. A schematic of these measurements are depicted in Figure A2.3.1. The main obstacle in this project is the fabrication of the 3D wave substrate. We have chosen to employ grayscale photolithography in conjunction with replica molding technologies to produce quasi
three-dimensional substrates. Many different fabrication techniques were attempted because we initially wanted to use fluorescence microscopy to analyze the above-mentioned neuronal properties. We found very few materials and conditions were not highly fluorescent. We began with a prepolymer polyurethane, Norland 68 optical adhesive, used by Mrksich et al. to produce quasi three-dimensional substrates.\textsuperscript{17} We cured the molded polymer with long wave ultraviolet light (385 nm), set the polymer at 50 °C overnight, and plated CHO-K1 cells to test for cytotoxicity. All of the cells in contact with Norland 68 died. To circumvent this issue, we deposited 50 Å Ti/150 Å Au onto the substrate and functionalized the surface with hexadecanethiol prior to protein application and cell plating. The substrates were now non-toxic, however, they were highly fluorescent. The inherent fluorescence of the polyurethane could not be mitigated through the addition of a fluorescence quencher to the polymer, either India Ink or Sudan Black B.

\textbf{Figure A2.3.1.} Schematic of a neuron growing on a three-dimensional wave substrate. Two properties that can be measured are the vertical angle of neurite growth as well as the angle of neurite growth from normal.
To address the issue of high fluorescence, we turned to Solvent-Assisted Molding (SAMo), developed by Lee et al.,\textsuperscript{18} to produce robust, non-fluorescent wave substrates (Figure A2.3.2.). We prepared a poly(methyl methacrylate) (PMMA) solution and applied it to an APTMS functionalized glass coverslip, a PDMS stamp was immediately placed on the PMMA and weighed down for eight hours. As the solvent evaporates, the polymer chains intertwine and a solid, patterned substrate is formed. In addition, PMMA is non-toxic and is stable under cell culture conditions. We were able to obtain z-stack images and created a volume view to look more closely at a neuron growing across a 20 µm tall wave (Figure A2.3.3).

**A2.4 CONCLUSIONS**

After much discussion, we realized that fluorescence microscopy would not provide the rigorous data we were aiming for because it would not allow for both the neurons and the pattern to be visualized together. We have now turned our attention to other methods of
imaging and analysis. Eventually, scanning electron microscopy (SEM) proved to offer the most promising results. Because we no longer need to worry about fluorescence properties, we returned to our initial work combining the molding of Norland 68, Ti/Au coating, and hexadecanethiol functionalization. Neurons growing on these substrates will then be fixed, dehydrated, dried using a critical point dryer to maintain proper morphology, and sputter-coated with gold. SEM images will be analyzed to determine the angle of neurite growth along the 3D wave pattern.

**Figure A2.3.3.** E18 mouse hippocampal neuron growing across a 20 µm tall wave pattern. The left panel depicts a volume image of a neuron growing across a wave. The neuron in the lower left corner is the same as that shown on the right. On the right, open arrowheads denote the cell body located at the lowest point of the pattern, while solid arrowheads point to the neurite crossing the apex. Scale bar is 100 µm.
A2.5 MATERIALS AND METHODS

A2.5.1 Grayscale Photolithography

One inch square microscope slides were cleaned in piranha solution (7:4 concentrated sulfuric acid: 30% hydrogen peroxide) for 2 h, rinsed thoroughly with deionized water, and dried with nitrogen. **Safety Note: Use extreme caution when working with piranha solution; it is highly corrosive and explosive when in contact with organic solvents.** Substrates were dehydrated prior to application of SU-8 2075 using a Cee 200CB spin/bake system (Brewer Science, Rolla, MO). The following spin program was used:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Spin Speed (rpm)</th>
<th>Acceleration (rmp/s)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>300</td>
<td>30</td>
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<tr>
<td>3</td>
<td>800</td>
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<td>5000</td>
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<td>5</td>
<td>2500</td>
<td>1000</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>1000</td>
<td>1</td>
</tr>
</tbody>
</table>

The resist-coated glass substrate was soft baked at 95 °C for 10 min. A grayscale bitmap file was converted to an LDF file for use with the direct-write LW325 LaserWriter system (Microtech s.r.l., Palermo, Italy). The substrate was patterned using this system followed by a post exposure bake at 95 °C for 7 min. The patterned substrate was developed in propylene glycol monomethyl ether acetate (PGMA) and rinsed with isopropanol.

A2.5.2 Substrate Fabrication

Sylgard 182 (Dow Corning) was mixed 10:1 (resin:hardener) and poured over the patterned master. The polymer was degassed using a vacuum dessicator and cured at 70
°C for 2 h. The final stamp was separated from the master and cut to size. The polydimethyl siloxane (PDMS) stamp was used to produce a variety of substrates for cell culture. These techniques are outlined in Table A2.5.1. Norland 68 Optical Adhesive must be cured with long wave UV light for 20 minutes followed by a 50 °C cure overnight prior to post-molding modifications. For PMMA patterned substrates, 3-Aminopropyltrimethoxysilane (APTMS) functionalized glass substrates were formed by covering a clean glass coverslip with a 10 mM APTMS in toluene solution and annealing at 150 °C for 15 minutes. The coverslip was rinsed twice with ethanol and dried with nitrogen. A few drops of 15% wt poly(methyl methacrylate) in toluene were placed on the APTMS surface and a PDMS stamp was immediately positioned on the PMMA solution. A weight held the stamp in place and the solvent was allowed to evaporate for at least eight hours prior to stamp removal.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Material (on glass coverslip)</th>
<th>Post-Molding Modification</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Norland 68 Optical Adhesive (Norland Products, Inc., Cranbury, NJ)</td>
<td></td>
<td>High Fluorescence Cytotoxic</td>
</tr>
<tr>
<td>2</td>
<td>Agarose</td>
<td></td>
<td>Unstable under cell culture conditions</td>
</tr>
<tr>
<td>3</td>
<td>Norland 68 Optical Adhesive</td>
<td>50 Å Ti/100 Å Au 10 mM hexadecanethiol soak for 1 h</td>
<td>High Fluorescence Non-toxic</td>
</tr>
<tr>
<td>4</td>
<td>Norland 68 Optical Adhesive + India Ink</td>
<td>50 Å Ti/100 Å Au 10 mM hexadecanethiol soak for 1 h</td>
<td>India ink does not dissolve in Norland 68, appears ‘spotty’ in fluorescence images</td>
</tr>
<tr>
<td>5</td>
<td>Norland 68 Optical Adhesive + Sudan Black B</td>
<td>50 Å Ti/100 Å Au 10 mM hexadecanethiol soak for 1 h</td>
<td>High Fluorescence</td>
</tr>
<tr>
<td>6</td>
<td>Polyacrylamide</td>
<td></td>
<td>Did not remain bound to glass under cell culture conditions</td>
</tr>
<tr>
<td>7</td>
<td>10 mM APTMS functionalization/SAMo using poly(methyl methacrylate)</td>
<td></td>
<td>Minimal fluorescence Non-toxic</td>
</tr>
</tbody>
</table>

Table A2.5.1. Parameters for the fabrication of 3D wave substrates.
A2.5.3 Cell Growth and Imaging on 3D Wave Substrates

CHO-K1 cells or E18 mouse hippocampal neurons were prepared and seeded onto patterned substrates as demonstrated in chapters three and five. Cells were cultured at 37 °C, 5% CO₂. At four days in vitro, cells were stained with MitoTracker Red 580 and fixed in a 3.7% paraformaldehyde/PHEM buffer for fluorescence imaging. Alternatively, for scanning electron microscopy (SEM), cells were fixed in 2.5% glutaraldehyde/Dulbecco’s Phosphate Buffered Saline (DPBS), followed by 1% osmium tetroxide/water. They were dehydrated for 10 min in 50%, 70%, 90%, 3X100% ethanol/water soaks. The dehydrated cells were then dried in a critical point dryer and sputter-coated with gold.
A2.6 REFERENCES

1. Clark, P.; Connolly, P.; Curtis, A. S. G.; Dow, J. A. T.; Wilkinson, C. D. W.,


