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USER-DEFINED PATTERNING OF NEURAL PROGENITOR CELLS ON 3D MICROPILLAR ARRAYS USING ROUND CROSS-SECTIONAL GEOMETRY, SPECIFIC DIMENSIONS AND THIOL-BASED CHEMICAL ADHESION

by

ANDREA SUZETTE WESSER
B.S. University of Central Florida, 2004

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Mechanical, Materials and Aerospace Engineering in the College of Engineering and Computer Science at the University of Central Florida Orlando, Florida

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ABSTRACT

The ability to control stem cell functions, particularly neuronal progenitors, has long since been believed to be the key to successful treatment of neurodegenerative disorders such as Alzheimer’s, Parkinson’s and accidents involving head trauma. The neurology field calls for many new solutions to address the controlled neural stem cell seeding and placement of cells for neural tissue regeneration. Self-assembled monolayers (SAM) from the alkanethiol group provide a straightforward applicable, reliable treatment for cell adhesion. An ODT/gold treatment was used to adhere the cells to patterned areas, due mainly to a high confluence of cells attracted to it, as well as the viable environment it produced for the cells. Arrays of micropillars, made of SU-8 photoresist, then covered with a thin film of gold and treated with the ODT, created scaffolding allowing manipulation of neural stem cells. Based on multiple trials of observing varying cross-sectional geometric parameters, metal layer thicknesses and the ODT/Gold treatment, this study explores seeding density control, base and circumferential cell population dependence on those parameters.
I dedicate this final culmination for the years of support and love given to me during my education. This work is for those who have encouraged me to succeed: my mother, Christine Boryszewski, my father, David Wesser, my brother, Evan Wesser, my best friend and love of my life, Leonardo Rocha and my closest friend Rebecca Whyte.
ACKNOWLEDGMENTS

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<tr>
<td>ACSF</td>
<td>Artificial Cerebrospinal Fluid</td>
</tr>
<tr>
<td>BOE</td>
<td>Buffered Oxide Etch</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized Water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diamine Triacetic Acid</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>MHO</td>
<td>16-mercaptohexadecanol</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<td>MUA</td>
<td>Mercaptoundecanoic Acid</td>
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<td>NCAMS</td>
<td>Neural Cell Adhesion Molecules</td>
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<td>ODT</td>
<td>Octadecanethiol</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PECVD</td>
<td>Plasma-Enhanced Chemical Vapor Deposition</td>
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<td>PGA</td>
<td>Polyglycolic Acid</td>
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<td>PLA</td>
<td>Polylactic Acid</td>
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<td>PMMA</td>
<td>Polymethacrylate</td>
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<tr>
<td>SAM</td>
<td>Self-assembled Monolayer</td>
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<td>TCE</td>
<td>Trichloroethylene</td>
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INTRODUCTION

The ability to control stem cell functions, particularly neuronal progenitors, has long since been believed to be the key to successful treatment of neurodegenerative disorders such as Alzheimer’s, Parkinson’s and accidents involving head trauma. In a study done by B. Reubinoff et. all in 2001, the use of neuronal progenitor cells for neural tissue regeneration was proven effective: inevitably, they could regrow tissue, differentiating into the three neural lineages of astrocytes, oligodendrocytes, and mature neurons. Upon transplanting the neural stem cells into newborn mouse brains, the group observed the cells entering the host brain parenchyma, spreading throughout and differentiating into the three neural lineages. The transplanted cells infiltrated the host brain and differentiated in a region-specific manner, indicating that they could respond to local cues and participate in the processes of host brain development [1].

However promising, by studying the nature of these stem cells, upon implantation, a more precise functionality can be predicted. In an overview in 2007, M. Mehler et. all demonstrated that exactly which lineage the cells would become depended on developmental mechanisms [2]. They explained further that ‘selected’ cells to survive would receive environmental signals to encourage proliferation [2]. For instance, specific to the proper development of neurons, neurogenesis initiates a sequence of growth activity where radial glia form a scaffold that enables neuroblast (neuron precursor) migration [2].

Cell survival is indeed, inherent on support structures, far beyond just for the purposes of migration. Many have noted the importance of Neural Cell Adhesion Molecules (NCAMs) and
matrix metalloproteinases (MMPs) as essential to neural cell survivability. C. Johnson et. all have identified which events (such as polysialylation) could obliterate NCAMs and lead to cell membrane weaknesses, an inability to adhere in the host tissue and inevitably, apoptosis [3]. A. Page-McCaw et. all found MMPs significant to intracellular junctions [4]. And though MMPs appear to allow embryonic development in its absence, mutations of particular types being withheld from rat embryogenesis brought about several findings: central nervous system developments were found altered and tubular structures (trachea, blood vessels, brachia) were found to be defective [4].

Most interesting to the abundance of neural progenitor cell studies, however, is the big question for neural tissue regeneration: can neural stem cells entirely regenerate lost tissue? To this very question, several neurologists looked toward older efforts in stimulating neural growth before the advent of stem cells: synthetic bio-scaffolds. Since the 1970’s, a number of materials were evaluated and over the last three decades, have become innovative and useful, causing some growth. Today’s popular biocompatible polymers, for a wide range of medical purposes, include polylactic acid (PLA) and polyglycolic acid (PGA). The material has demonstrated few biocompatibility issues and is even degradable, allowing tissue healing and growth without permanent placement or further complications for years to come [5].

It did not take long for a number of neurologists to pair the two worlds together and along with the scaffold plus neural progenitor cell trials, compare the regeneration effectiveness against controls of scaffold-only and neural stem cells-only. E. Snyder and his team, found effective results when PGA scaffolds are combined with neural progenitor cells: vascularization
developed within two weeks; no gliotic scarring occurred; host and donor cells began to connect processes from both directions; and deep tissue recovery could be seen after six weeks (see Figure 1) [6].

Figure 1a demonstrates acute rabbit brain necrosis while Figure 1b shows slight tissue growth immediately upon PGA scaffold+NSC implantation (black fibers are PGA scaffold); Figure 1c demonstrates full neural tissue regeneration, as does Figure 1d, which features the site of Figure 1b with degrading PGA scaffold fibers, both after six weeks [6]. Furthermore, additional tests on whether motor skill recoveries were made possible were conducted and they too were found to be re-enacted [6]. The controls that were conducted, whether PGA scaffolds alone or neural stem cells alone, could provide identical results, proved that the combination showed the most potential for proper neural regeneration efforts [6].
Figure 1 Success of NPC and scaffolding combination: (a) acute rabbit brain necrosis, (b) site with immediately applied scaffold+NPCs and nearby tissue death, (c) full neural tissue growth 14 days later; (d) complete growth at same site as (b) [6]

In another solution with similar implications, G. Silva et. al used a network of nanofibers that assemble in vivo, producing a viable scaffold [7]. This scaffold in particular, was found to explicitly derive neurons as opposed to astrocytes, which do not allow full neural functioning when compared [7]. Though 5 – 8 nanometers in diameter, and lengths three to four orders of magnitude larger, the nanowire networks offered high aspect ratios and therefore, more surface area for cells to infiltrate; far more than a natural extracellular matrix [7]. Cell densities were higher than any other method and differentiated in seven days, but caused noticeable clusters, or
neurospheres, which became so populated in the center that cells starved of nutrients and oxygen, thus leading to cell death [7, 8].

The neurology field calls for many new solutions to address the controlled neural stem cell seeding and placement of cells for neural tissue regeneration. Though these previous studies have showed tremendous promise in tissue re-growth, and certainly demonstrated the clear conclusion of the maximum effectiveness of combining neural stem cells with scaffolding, there is much more to explore. Precise control of cell seeding densities in addition to the control of where exactly cells are seeded on the scaffold, could potentially lead to specific directionality and particular therapy placements. However, as proven in the scaffold plus neural stem cell experiments mentioned above, the need for developing a three-dimensional scaffold was apparent.

A variety of 2.5 and 3-dimensional scaffolds have been used to handle macro-samples of tissue as well as cell lines. In 1991, G. Picha designed and patented a micropillar array, rectangular and cylindrical, that would diffuse nutrient rich or deliver drugs when interfaced with host tissue [9]. Very similar in concept, in 2003 Ph. Passeraub et. al created a microfluidic chamber with micropillar arrays at the bottom to allow a proper flow of oxygenated artificial cerebrospinal fluid (ACSF) to perfuse through a brain tissue slice [10]. Though the attempt provided mediocre nutrient diffusion, the amount of ACSF provided through the microarrays and chamber was not enough to keep the entire surface area of the slice healthy [10].

Many attempts to provide healthy implantations or maintenance of tissues in vivo through scaffolding would prove most beneficial if they contained cells rather than macro-scale tissue
systems. In 2007, S. Tao et. al. produced a deep-micropore scaffold of polymethamethylacrylate (PMMA) to seed with retinal progenitor cells and aid in retinal tissue regeneration [11]. Y. Toh et. al. mimicked Passeraub’s work in 2007, demonstrating perfusion-culturing of cells [12]. Utilizing a microfluidic channel with 3D disarranged structures (not pillar arrays), Toh was able to simulate the *in vivo* environment of bone marrow mesenchymal stem cells, thus leading to the cells retaining their cell-specific functions and differentiation competencies throughout the experiment [12].

The most notable combined use of micropillar arrays and stem cells, came from Y. Tanaka et. al. in 2006, when his group utilized cardiomyocytes to manipulate polydimethylsiloxane (PDMS) micropillars [13]. Fibronectin added to phosphate buffered saline (PBS) encouraged cell adhesion to the pillars [13]. As the cardiomyocytes pulsed, much like adult cardiomuscular cells, the pillars were pulled along with the contraction of the cell, thus producing a displacement of the pillar at the site of attachment [13].

Once controlled on a substrate via organized patterning and with outside stimulation, a precise manipulation of the cells may be achieved. The first of many steps in creating a manipulative scaffold for the precise control of unpredictable stem cell behavior is to pursue selective cell adhesion [14]. Various treatments of the surface have been studied over the last ten years, whether showing a preferential for cell adherence, such as organic polymers (i.e. photoresists), or to prevent cell adherence, such as specially formulated alkyl silanes [15]. Self-assembled monolayers (SAM) from the alkanethiol group provide a straightforward applicable, reliable treatment for cell adhesion. Alkanethiol SAMs, such as 11-mercaptopundecanoic acid (MUA)
[16] or octadecanethiol (ODT) [14], are preferred for producing strong bonds between gold and the thiol groups’ sulfur atoms, and then leaving the unpaired alkyl group’s carbon atoms to receive and bond strongly with any of the various biomolecules.

In previous unpublished work of the University of Central Florida Nanofabrication and BioMEMS Lab, a variety of substrates for patterning sites of alkanethiol self-assembled monolayers (SAM) were assessed to best host neuronal progenitor cells for potential biomedical applications [17]. The substrates, silicon, borosilicate glass, Plasma-Enhanced Chemical Vapor Deposition (PECVD)-grown silicon dioxide and PDMS, were all tested for inevitably, cell adhesion, electrical insulation, and further functions. An ODT/gold treatment was used to adhere the cells to patterned areas, due mainly to a high confluence of cells attracted to it, as well as the viable environment it produced for the cells.

As initial experiments developed, it was found that arrays of micropillars, made of SU-8 photoresist, then covered with a thin film of gold and treated with the ODT, created scaffolding allowing manipulation of neural stem cells. Based on multiple trials of observing varying cross-sectional geometric parameters, metal layer thicknesses and chemical treatments, the following study explores seeding density control, base and circumferential population dependence on those parameters.
MATERIALS AND METHODS

The materials used in preparing 2.5D and 3D scaffolds of micropillars require a selection process that covers many important characteristics such as: *in vitro* biocompatibility to avoid cell toxicity; height limitations of specific photoresists; rugged binding SAM for a multi-layer fabrication process and easily contaminated gold surface. Taking considerations for both microfabrication needs as well as biological-driven experimentation requirements produced an exhaustive examination of materials, handling and fabrication techniques necessary to the success of this study.

The testing apparatus fabrication, up until final cellular observations, consists of three main scientific concentrations: microfabrication techniques inherited from the semiconductor industry to produce micropillar arrays; the nanofabrication technique of surface modification utilizing an alkanethiol based SAM for cell adhesion; and the latest neuroscience technique for neural tissue regeneration via neural progenitor cell *in vitro* scaffold adhesion/survival studies. For each concentrated sub-category of the study, a series of varying materials and methodologies were assessed.

For the fabrication of micropillar arrays, though a number of photoresists are available, MicroChem’s SU-8 50 was selected for achieving the exact height of 100 microns. The height was selected in proportion to the cells soma size, which range from 10 microns to 20 microns. Another purpose for using SU-8 was in utilizing its tendency to remain attached to the substrate regardless of traditional photoresist removal methods, if properly utilized through baking,
exposure and development stages [18]. As seen later in this section, the use of acetone to remove another photoresist does not affect the SU-8. Since SU-8 requires additional adhesion promoters if spun onto glass substrates, and since the promoters are toxic to the cells, the substrate utilized was mechanical grade silicon wafers, which do not require any adhesion promotion. Mechanical grade silicon wafers were used since the substrates act as a scaffold base and are not relied upon for their semiconductor properties.

As described in the introduction, an ODT SAM was selected as the main surface modification allowing cell adhesion. Unlike other alkanethiol SAMs, ODT SAMs demonstrate adhesion reliability from experiment to experiment. Furthermore, compared to other SAMs such as cysteamine, the ODT SAM does not require an Argon-rich environment for preserving its chemical integrity or is not considered hazardous to the user [19]. ODT’s self assembly begins as the thiol group (-SH) attaches to the gold coated SU-8 micropillars through a strong covalent bond with a high bond enthalpy of 418±25 kJ/mol [20]. From this thiol head, the ODT SAM possesses 17 carbenes (:CH\textsubscript{2}) and one methyl (-CH\textsubscript{3}) hanging group [21]. Its total formula is CH\textsubscript{3}(CH\textsubscript{2})\textsubscript{16}CH\textsubscript{2}SH. Figure 2 shows its molecule structure. An array of biomolecules, in this particular study, neuronal progenitor cells, can then be bound to this hanging methyl.

Figure 2 Octadecanethiol molecule
Van der Waals interactions, steric relationships, and electrochemical interactions between the alkyl heads of the ODT molecules forced the ODT molecules to jettison out from the gold and sulphur bonding sites resulting in parallel ODT processes extending outward from the substrate [21].

Since the study was in collaboration with the University of Central Florida Neuroscience Lab, which specifically utilizes neural cell lineages, the cells used were determined through this relationship. SH-SY5Y cells, third generation neuroblastomas, were used throughout the study. Tumor cells are often utilized for their fast proliferation (2 – 3 days for high confluences to appear) and for their functional similarities to non-tumor neural stem cells. Like non-tumor neural mesencymes, they tend to cluster and can be differentiated with the use of retinoic acid.

The substrates were fabricated in similar procedures over a number of controls. To provide the necessary experimental conditions, the following mechanical and chemical controls were provided:

a) Silicon wafer with SU-8 pillars only  
b) Silicon wafer with SU-8 pillars, 90° metal deposition, and ODT  
c) Silicon wafer with SU-8 pillars, 45° metal deposition, and ODT  
d) Silicon wafer with SU-8 pillars, two 45° metal depositions for complete pillar coverage, and ODT  
e) Control groups b-d without ODT self-assembled monolayer added  
f) Silicon wafer without SU-8 pillars but with metal depositions and ODT
g) Silicon wafer with SU-8 pillars, metal deposition, and ODT with circular pillars only less than or equal to 450 µm in diameter

h) Silicon wafer with SU-8 pillars, metal deposition, and ODT with pillars of different shapes, diameter, and proximity to other pillars

i) Silicon wafer with micropillars of SU-8, metals and ODT ONLY; with the silicon base bare

Though both the circular and varied cross-section shaped pillars were fabricated in the same method, the design for the mask of the different shaped pillars was specifically designed as a continuation of study upon a previously created circular pillar array mask. Since the original, circular pillar array of varying cross-sections 80 to 300 micron in diameter, Figure 3a shows the overall mask design used to pattern and assess pillars of varying cross sectional geometries as well as diameters from 400 micron to 1.2 millimeters. Figure 3b shows a magnified view of pillars used to assess non-circle, rounded geometries and 3c shows a magnified view of those pillars used to demonstrate angular geometries

Every silicon wafer started through the same cleaning process. The wafers were first submerged in a buffered oxide etch (BOE) containing ammonium fluoride and hydrofluoric acid for 15 seconds to remove any oxides that had accumulated on the wafer. Following their submergence in BOE, a trichloroethylene (TCE) wash followed by washes with acetone, methanol, and deionized (DI) water complete the cleaning. A nitrogen spray was then employed to remove all water droplets remaining on the wafers. The wafers were then baked at 150°C for 10 minutes to dehydrate.
For the wafers with SU-8 pillars, SU-8 50 photoresist was spread on wafers at 500 rpm with an acceleration of 100 rpm/s² for 10 seconds and then spun at 1000 rpm with an acceleration of 300 rpm/s² for 35 seconds, to achieve a thickness of 100 microns. Since the SU-8 is very viscous and thick, the centripetal force generated by the spin coater created a bead edge, which was removed with a makeshift tool. The wafers were soft baked at 65°C for 10 minutes and then at 100°C for 28 minutes (see Figure 4a).

For exposure, a Karl Suss mask aligner was used with a dark-field-light-features mask to pattern the pillars and create 3D structures. Following alignment, the wafers were baked for 1 minute at
65°C and then for ten minutes at 100°C. They were removed from the oven and allowed to cool to room temperature (22°C). The wafers were then developed in SU-8 developer for approximately 7 minutes a piece and remaining SU-8 residue was removed using isopropanol. Finally, the wafers were washed with methanol and DI water to remove all remnants of isopropanol and unmarked photoresist. The wafers were then dried using a nitrogen spray (see Figure 4b).

During the metal deposition, a layer of titanium to promote adhesion of the gold, followed by a layer of gold, was deposited via thermal evaporation in a multi-source system. There are a variety of metals that can be used to adhere gold to silicon, however, titanium was chosen since it is considered biocompatible, and indeed, showed no signs of affecting the cells negatively. The way the wafers were oriented in the chamber, suspended from the plenary wafer holder, enabled evaporation paths of direct-on (Figure 4c), approximately at 45° (see Figure 4d) and > 75° (Figure 4e) to the metal sources to achieve a variety of metallic deposition scenarios. Titanium was evaporated at 112.5 amperes while gold was evaporated at 83.6 amperes allowing for ionic bonding between the titanium particles and silicon wafers, and a metallic bonding between the titanium and gold. The process was doubled for complete pillar metallic coverage, following the 45° angled deposition (Figure 4f).

For gold “island” micropillar arrays, in which the base of the silicon is no longer coated in gold, but is bare silicon, there is another fabrication procedure.
Figure 4 Fabrication of SU-8 micropillars (non-Island type) and preparation for cells: (a) SU8 spun on, (b) pattern SU8, (c-f) gold deposited directly-on, at 45, above 75 and at 90 degree angles, and double deposited, (g) ODT assembled
With the gold coating of either pillar array types, the alkanethiol compound, ODT, could now be used to culture neural mesenchyme. The wafers are then washed with 200 proof ethanol. Next, the ODT solution was prepared, vigorously mixing 2mM of ODT in the ethanol. The ODT solution was then poured over the wafers and allowed to evaporate for 24 hours, causing chemisorption reactions to occur between the ODT’s sulfahydryl groups and the gold deposited on the wafer (see Figure 4g). The additional surface area acquired through these intermolecular forces aid in cellular adhesion due to increased area for glycoprotein (often integrin-fibronectin complex) attachments [21].

Following wafer preparation, the cell sub-culturing process must be initiated. Before introducing cells into the lab environment, a septic technique was performed to sterilize the laminar flow hood under which all cellular subculturing and maintenance would be completed. The SH-SY5Y neural tumor cell line was maintained by bi-weekly supply of a pre-made mixture consisting of growth media (buffer and salt solution to maintain pH and tonicity), Fetal Bovine Serum (nutrition), and an antibiotic/antimicotic. SH-SY5Y cells were seeded at 5 X 10^6 cells per 75 cm^2 tissue culture treated flask (Corning). Cell culture media was Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologics) and 1% anti-mycotic/anti-biotic (Invitrogen). When appropriate, the cell lines were subcultured by first discarding used media from the cell flask.

The cells were then trypsinized twice: the first treatment was intended as a rinse to deactivate any remaining media and the second treatment was intended to cleave the self-adherence of cells off the bottom of the flask over a 1.5 minute incubation period in a carbon dioxide incubator.
Following incubation, the trypsinized cells along with 10 milliliters of new media were centrifuged at 1500 rpm for 3 minutes and the resulting supernatant was discarded. Re-suspended in fresh media, the cell suspension was allocated in 1 milliliter quantities to new flasks to allow for continuation of the cell line.

SH-SY5Y cells were cultured on the microscaffolds once a stable cell reservoir had been established through successive divisions and allocations. Prior to subculturing, the wafers were incubated under ultraviolet light for 1 hour and sterilized with ethanol. Cell flasks to be cultured were then removed from storage, sterilized, and placed under the laminar flow hood. Cells were then trypsinized, centrifuged, and allocated into 10 milliliter suspensions for culturing on the microscaffolds. The allocates were then poured into petri dishes containing the microscaffolds (including control groups) and allowed to incubate in an incubator, maintained at a humidified atmosphere of 5% CO$_2$ and 37°C. SH-SY5Y cells were passed twice a week by trypsin/EDTA (Ethylene Diamine Triacetic Acid, Invitrogen) treatment. Cells were plated on gold patterned pillar arrays in 35mm petri dishes at a seeding density of 1X10$^5$/cm$^2$.

CellTracker (Molecular Probes) was applied to cells prior to plating at a concentration of 15μM. Media was replaced 24 hrs post cell plating and monitored under an inverted fluorescent microscope. Imaging was done using an inverted fluorescent microscope (Leica DMI 6000B) with DIC. For fluorescent microscopy a DAPI/Rhodamine/ Fluorescein Isothiocyanate (FITC) filter cube was utilized (Chroma). CellTracker dye (Molecular Probes) was visualized by use of FITC filter with emission/excitation of 480nm/528nm.
Image analysis was done using Openlab 4.0.1 software (Improvision) to assess cells dependence on pillar characteristics as described previously. The cells are observed after 48 – 72 hours of incubation. The images went through cell counting, done graphically as well as supplemented by the National Institute of Health’s ImageJ software, a visual analysis tool which differentiates threshold contrast values to define regions of interest, such as the SH-SY5Y cells. The images selected for this study and the plots that follow contain values averaged over numerous different test substrates spanning fourteen months of experimentation and randomly chosen for the variety of experiments that follow in the Results section. Once analyzed, the data was maintained and plotted by Excel, utilizing built-in trend and other modeling devices to demonstrate cellular patterns and pillar characteristic relations.

Scanning electron microscopy (SEM) was also used to view cell growth. Immediately upon removing cell media, the substrates with still-live cells were covered with a thin layer of gold-platinum via sputtering. The metallization was necessary to view the cells under SEM. A Hitachi S3500N SEM allowed a wide range of viewing opportunities, including image capturing of the pillar sides.
RESULTS

In the initial experimentation for selecting a thiol-based SAM to selectively adhere cells, it was found that ODT, indeed, produced tangible results in which patterns emerged where defined to be, while demonstrating a feasibly reproducible procedure. Figure 5a shows ODT treated gold features populated by stem cells (green fluorescence) while surrounding non-gold surfaces show few to no cells adhering. Figure 5b shows the control, in which no ODT treats any of the same surface. Furthermore, Figures 5c and 5d demonstrates the minimum feature resolution (the smallest feature here is 8 micron) this adhesion method can accommodate. (All scales shown are 65 micron unless otherwise noted).
Figure 5 ODT treatment shows defined cell adhesion: (a) ODT adhered cells, (b) same feature with no ODT and no cell adhesion, (c - d) 8 micron feature cell adhesion (all scales shown are 65 microns)
After the ODT proved to selectively pattern cells, the same needed to be demonstrated on 3D scaffolds for potential tissue regeneration therapy applications. Figures 6a and b demonstrate the micropillar arrays with ODT to promote cellular adhesion and without ODT, respectively.

Figure 6 Micropillar with (a) ODT and (b) without (all scales shown are 65 microns)

As can be observed in Figure 6a, a conformal and dense cellular population can be witnessed around the pillar and its base. It should be noted, that though the cells are dyed by the green fluorescence, the SU-8, which does not fully encapsulate the pillar (its single deposited), absorbs some of the dye. At the top of the pillar of Figure 6a, on its circumference, however, a dense pack of cells can be seen indicated by a large population of green fluorescence. This indicates that the cells have climbed the full height of the pillar (100 micron). In Figure 6b, where there is no ODT, and therefore, nowhere for cells to attach, the cells have preferred the bare silicon (since the gold is not deposited on the side of the pillar that was not exposed to the deposition
source). Here, the cells are forced to find attachment, or as described in the introduction, they will die.

Further along with this same experiment, unique patterns such as those in Figures 7a-d, indicated preferential patterning tied to geometric parameters such as dimension and shape, though gold treated with ODT was everywhere. In Figures 7a and 7b, there is a definitive base pattern that emerges, sectioning off substrate cell populations by inducing self-made boundaries. In Figure 7c, the smallest pillars of 80 micron diameter show few traces of highly populated bases, but densely populated circumferential cell population indicated by the asymmetric, green fluorescence around its perimeter. For other geometries, such as Figure 7d, angular shapes, demonstrate that cells prefer corners, where two treated surfaces may incite better adhesion.
Figure 7 (a-d) Unique patterns of cells emerge on pillar arrays (all scales shown are 65 microns)
After these initial experiments, a study was launched to determine any correlations between these unique patterns. The next experiment looked for a relationship between circular pillar dimensions and cell populations. Figures 8 a – f show the range of diameters, from 100 micron to 1.1 millimeter and their influence on base populations. As the Figures go from smallest to largest in diameter, there is a strong suggestion that the cell population at the base not only dwindles to smaller numbers, but does not or cannot cover the entire base perimeter. One theory for this is that, should the pillars choose a site to anchor then “spiral” in proliferation around the circumference, the radius of curvature, as it gets larger, presents a challenge for cells to ‘reach’ the next anchor location.

Figure 9 demonstrates the relationship between the pillar diameters and cell base population (multiplied by 5 to average out sites of intense fluorescence which are clusters of 2 – 10 cells) within 25% of the diameter’s length from the pillar wall (i.e. Figure 8c, a pillar of diameter 350 microns, shows cell population preference within 88 microns of the pillar wall), obtained from five separate experiments and with at least three cell counting iterations. The smooth trend line (shown in black) contains a peak in base population immediately from the pillar at 250 micron diameter pillars. The same relationship was studied, however this time, with the cell base populations that lie between 25% - 50% of the pillar diameter away from the pillar wall. The dotted trend line (shown red) in Figure 9 shows the maximum peak shifted to the left, indicating a tendency for further-from-the-pillar base growth.
Figure 8 Circular micropillar diameters and base populations: (a) 100 um, (b) 250 um, (c) 350 um, (d) 450 um, (e) 750 um, (f) 1.1 mm (all scales shown are 65 microns)
The relationship between the pillar diameters and cell circumferential (directly-on wall) populations were also assessed from Figures 8a-f. The pillar circumferential population was assessed via a polar graph as seen in Figure 10. The graph demonstrates the degree of coverage (from $0^\circ$ – $360^\circ$) of the circumferential length of the circular pillar that has been populated by cells. Since a unique pattern emerged in three distinct trials, all matching these results, it is evident that the trend for this relation shows complete coverage of the pillar perimeter occurs in smaller diameter pillars (less than 250 micron).

Figure 9 Diameter versus base cell population (smooth line - 25% within pillar wall; dotted line 50% within pillar wall)
Other geometric parameters, such as cross sectional shapes, round versus angular, were assessed. Each substrate contained a variety of different cross sectional-shaped pillars. These included angular shapes such as triangles and stars, as well as rounded shapes such as spirals and clubs. Figure 11 demonstrates base cell counts compared across the different cross-sectional
geometries. Though the rounded cross sections seemed more populated along the perimeter, the angular cross sections yielded clusters of the cells in the corners.

Figure 11 Base cell count versus cross sectional geometry

To observe the cells up along the pillars lengths, since the fluorescence imaging only shows the top-view of the pillars, scanning electron microscopy (SEM) was utilized. Figures 12a - d, demonstrate unique views of the pillar arrays including pillar groups not seen by the fluorescence imaging (a,b), as well as pillar wall texture (c) and cell clusters at base of pillars (d). The SEM also allowed imaging of a relationship between pillar diameter and the extent of wall climbing.
patterns of cells, which did emerge in a distinctive pattern. Figures 13a – f, show this unique pattern in wall climbing.

Figure 12 SEM images of pillar arrays (a,b), side wall texture (c), and cell clusters at the base (d)
Figure 13 SEM images of wall climbing patterns emerged from the cell attachment - (a) 100 um, (b) 250 um, (c) 450 um, (d) 750 um, (e) 950 um, (f) 1.1 mm
Figure 14 demonstrates the average height of cell-attached wall measured from the pillar base as related to the diameters of the pillars. This shows a tendency for the cells to climb the full height of 100 microns with the smaller diameters. The plot contains values averaged over numerous different test substrates spanning four months of experimentation and randomly chosen for three separate sessions with the SEM.

Figure 14 Pillar diameter versus height of cell-attached wall
In the following Discussions section, the above images and plots will be explained in further detail and suggestions as to why the following phenomena were found as such, will be rationalized.
DISCUSSION

In the order with which this study was conducted, it is important to first note the significance of the ODT based SAM as the adhesion method with which the cells could be patterned on the surfaces. In discussions with materials and nanotechnology experts, a major breakthrough in traditional biomolecular adhesion techniques was brought forth in this study. In a typical SAM-based technique for biomolecule-artificial substrate adhesion, a hydrophilic head, such as a hydroxyl group, must be utilized in order to properly bond the two. Most especially in the scenario where adult neural cells are used, a bond occurs as the charge between the cell, whose membrane is negatively charged, and the hydrophilic head of the SAM, positively charged, are attracted each other. However, it was determined that, although the bonds between the neural progenitor cells in this experiment and the ODT SAM remain, the hydrophobic methyl head, already neutral, should not offer any promotion of adhesion.

Along this line of thought, an additional experiment was conducted to determine whether a hydrophilic SAM would also prove the proper adhesion and thus show some error in the production of the ODT SAM upon the substrate. The hydroxyl-terminated SAM of 16-mercaptohexadecanol (MHO, HS(CH$_2$)$_{16}$OH) was left to incubate in a similar manner as the ODT, as described in the Materials and Methods section, though this time with a 1mM solution of the MHO in 50% aqueous ethanol (200 proof) [22]. Gold coated substrates of ODT versus MHO were assessed via contact angle measurements. Rame-hart contact angle measurement device was utilized along with the DROPImage Advanced software program to analyze the approximate contact angle. The untreated glass portion was first tested, showing a contact angle
of 66° (Figure 15a). Figure 15 demonstrates the difference in contact angles, and as assumed the ODT treatment shows a hydrophobic nature, with a contact angle of 108° (Figure 15b) while the MHO proves a hydrophilic nature with a contact angle of 35° (Figure 15c).

![Figure 15 Contact angles of a water droplet on a substrate: (a) before surface treatment and after treating with (b) ODT, and (c) MHO.](image)

After the contact angles were determined, the cells were plated on the substrates as described earlier in the Materials and Methods section. Figures 16a-e show a preference of the cells for the ODT treatment (Figures 16a-b) over the MHO treatment (Figures 16c-e; shadowed, darker region is gold). From the MHO derived substrates, it can be seen that the cells prefer the glass over the gold MHO-treated surfaces (16c-d) and the cells do not pattern with the gold features treated with MHO either (16e). It was found that, indeed, the hydrophilic SAM does not promote adhesion with the neural progenitor cells used. Though further tests would need to be conducted to elaborate on this theory, there is evidence that suggests the neural progenitor cells prefer the hydrophobicity of the ODT SAM because either their membrane holds other charges or there are other bonds, perhaps mechanical, that initiate the same bonding mechanism.
Figure 16 (a-b) Cells on features treated with ODT versus (c-e) cells on features treated with MHO
Another experiment to also check on the correct production of the ODT-formed SAM included a slight difference from the method described in the above Materials and Methods section: rather than remove the lid from the substrate in ODT solution during incubation, thus allowing evaporation of the solution entirely, the lid was kept on the Petri dish and after the same 24 hour incubation period, the substrate was removed and cleaned in the original method. The rationale for this included the fact that such long molecules like ODT, with 16 repeating carbenes along each chain, either bent over, thus producing peaking parts of the molecules of carbene not the terminating methyl group, or that a quick evaporation of the solution produced a non-uniform SAM. In order to determine that there was no significant difference in the incubations or proper development of the SAMs, contact angle measurements of the two, post-incubation and ethanol wash, were taken. And here too, it was found, that the difference in incubation methodology was irrelevant: both showed consistent contact angle values (see Figure 17a-b). Figure 17a shows the hydrophobic nature of the ODT quickly evaporated at a contact angle of 108° versus the ODT incubated in solution with a similar angle of 111°, shown in Figure 17b.
Figure 17 Contact angles of a water droplet on a substrate with (a) ODT incubated without a lid versus (b) with a lid.

This indifference to preparation can also be found in work by R. Subramanian et. all on the surface characterization and kinetics of alkanethiol SAMs [23]. Figure 18 demonstrates that for 1mM of ODT in ethanol (plot a of Figure 18), complete uniformity, $\theta$, of ODT over the substrate surface is established within 17 minutes. Even with the lid off in these experiments while allowing ODT to evaporate, the substrate was still submerged fully in solution for over 30 minutes.
Figure 18 Uniformity of adsorption of ODT with respect to time and varying concentrations, (a) 1mM, (b) 20 um, (c) 5um, and (d) 1um [23]

As for the series of geometric relations to further cell patterning and growth, a number of theories come to mind. For instance, the strongest explanation for the inherent patterning and proliferation within areas of the pillar arrays and a dependence on diameter stems from the radius of curvature with respect to the cells’ size. It would appear that the smallest pillars of 100 – 250 micron in diameter, allowed the cells to “wrap” around the circumference because cells surrounding other areas of the perimeter were closer to each other. This would be more difficult for cells 10 to 50 times smaller than the pillar diameter, to proliferate towards other clusters were the radius of curvature so much larger.
Furthermore, the cells could more than likely climb up the wall because of a larger base population: the cells had less space to proliferate so they grow upwards. As for geometry specific dependency, the rounded pillar walls afford many of the benefits described above because of curvature. Although the cells found the inward corners of angular cross-sections appealing due to more surface area to attach to, perhaps outward corners (such as the points of the stars) made it difficult for the cells to proliferate towards each other.

During this study and as can first be seen in Figures 7a and 7b, the base populations of the cells introduced some curious behaviors. At the diameter of 250 micron only, although the entire substrate was treated with ODT on gold, a unique ring-like pattern of cells emerged at the base of the pillars. An additional experiment was conducted to simply replicate the unique, self-induced patterning the cells carried out for 250 micron diameter pillars, using the same fabrication, ODT preparation and cell treatment as used throughout this study. Figures 19a-b shows this unique pattern in the initial experiment as Figures 19c-d demonstrate the same results produced, this time in the replication study.
Figure 19 (a-b) Initial unique base patterning versus (c-d) replication of base unique pattern (all scales shown are 45 microns)
There are few explanations an engineering student can find to determine why the cells pattern in this manner. A plausible theory, though further studies with the assistance of cellular biologists and embryo-neurologists stems from the tendencies of neural progenitor cells to form microscopic tubular geometries. During the evolution of the human embryo, the neural progenitor cells are responsible for the formation of the neural tube which is the foundation of the spinal cord and ensuing tissues. Other evidence, such as that stated in the work of Page-McCaw et. al, includes the cell line’s recognized assistance in the formation of axons, other neural tissue systems and the trachea, all tubular organs, in fruit flies [4].
CONCLUSION

The patterning of neural progenitor cells on pillar arrays has its benefits. Long plagued by the inability to control these cells which show huge potential in neural tissue regeneration, such patterning in a three-dimensional structure allows the neuroscientist to precisely apply the neural progenitors as well as control proliferation. The precise control of geometric attachment can allow deep brain tissue re-growth if the cells are grown 360° about the pillars and implanted as such, or if grown along a fraction of the circumference, can be ideally used for brain tissue growth along the skull, as well as for spinal cord wall regeneration.

This study brings about a number of validations to the unpredictable behavior of neural stem cells. Observations included:

- Neural progenitor cells when patterned amongst circular pillars varying in diameter of 80 microns to 1.1 millimeters, and 100 microns in height, tend to proliferate more about the pillar base of those with diameters of 250 – 350 microns.

- Neural progenitor cells when patterned amongst circular pillars varying in diameter of 80 microns to 1.1 millimeters, and 100 microns in height, tend to proliferate about the entire circumference of pillars of diameters of 250 microns or less.

- Neural progenitor cells when patterned amongst circular pillars varying in diameter of 80 microns to 1.1 millimeters, and 100 microns in height, tend to
proliferate up the full length of the pillar sidewalls with pillars of diameters 250 microns or less.

- Neural progenitor cells favor patterning to gold features via the alkanethiol self-assembled monolayer of octadecanethiol.

- Neural progenitor cells are not sensitive to particular substrate roughness, when comparing glass, silicon or polydimethylsiloxane.

- User defined neural progenitor cell patterning requires both chemical and physical parameters of geometry specifications and ODT SAM preferential chemistry.

- Though forced patterning and manipulation was in effect, a number of unique occurrences, like self-induced patterning and the unlikely nature of these neural cells to adhere to hydrophobic surfaces, were found through many repetitions of the experiments.
FUTURE WORK

Future work on this study will include the overall use of micropillar arrays for a more focused neural tissue regeneration therapy. Currently, densely packed clusters of neural progenitor cells, known as neurospheres, are implanted at the sites of damaged neural tissue. By adhering to each other in a three-dimensional cluster, the cells are somewhat controlled and organized upon implantation. However, the spheres have two significant drawbacks: the cells become too dense as they proliferate in the center of the sphere, become cut-off from both nutrients and oxygen, and inevitably, die. As an alternative, these micropillars and their ability to promote proliferation in a three-dimensional direction over more surface area, will allow controlled, large populations of cells to remain vital. The use of such techniques as described in this study to pattern the micropillars, combined with bio-compatible materials to make the pillars from, will prove a viable concept in the ever-growing world of neural tissue regeneration therapies.
LIST OF REFERENCES


[23] Subramanian, R., and Lakshminarayanan, V. “A study of kinetics of adsorption of