Organization and Differentiation of Stem Cells on Delivery Scaffold for Retinal Tissue Engineering

by

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B.S. Mechanical Engineering, Business Economics and Management
California Institute of Technology, 2008

Submitted to the Department of Mechanical Engineering in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN MECHANICAL ENGINEERING AT THE MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2011

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Submitted to the Mechanical Engineering Department on May 18th, 2011 in partial fulfillment of the requirements for the degree of Masters of Science in Mechanical Engineering

ABSTRACT

Retinal degenerative diseases, including retinitis pigmentosa and age-related macular degeneration, affect more than ten million people in the US. Currently, there is no proven visually beneficial treatment for these types of disease; however, stem cell-based therapy is a recent strategy which has the potential to preserve and restore vision in these conditions. In addition to replacing lost or diseased cells, transplanted cells may be able to rescue dying photoreceptors of the host retina. While studies have shown that retinal progenitor cells (RPCs) delivered by bolus injection can differentiate into retinal specific neurons after subretinal transplantation, they have not been able to maintain morphologic development, lamination, or extensive integration with the host retina. Therefore, a mechanism is needed to confer organization and instructional cues to these grafted cells.

In this research, micro and nano-electro-mechanical systems (MEMS/NEMS) processing techniques were used to create biodegradable thin-film scaffolds to guide the differentiation and organization of stem cells for retinal tissue engineering. Through standard MEMS processes, including photolithography and reactive ion etching, a high throughput array of sub-micron features (500 nm to 1 µm) was fabricated into silicon wafers. A novel templating process was developed to then imprint these structures into biodegradable polycaprolactone (PCL) thin films (5 -10 µm) with minimal deformation to the imprinted features. PCL was chosen due to its low melt temperature, adaptability to microfabrication processing, as well as its mechanical and bioresorptive properties. Furthermore, PCL thin films have been shown to be well tolerated long term when transplanted in the subretinal space of mice. RPCs were cultured on PCL thin films, and cell responses to sub-micron topography of varying dimension and geometry were characterized using scanning electron microscopy and immunocytochemistry. Sub-micron features were found to definitively affect cell behavior. For example, while RPCs cultured on post structures demonstrated an early upregulation of differentiation markers, including rhodopsin and recoverin, RPCs cultured on a ridge-groove topography developed substantial elongation and parallel alignment in addition to upregulation. This unique structured PCL thin-film platform therefore provides a means to organize and differentiate RPCs in a controlled manner and offers potential as a clinical treatment for retinal degenerative diseases.

Thesis Supervisor: Carol Livermore
Title: Associate Professor of Mechanical Engineering
ACKNOWLEDGEMENTS

I would like to give my sincere gratitude to all the people that have helped me with my master's work.

First, I would like to thank Draper Laboratory for their superb resources, material assistance, and personnel for support of my thesis. Specifically, I want to thank the technical staff in the MEMSFAB (William Teynor and Connie Cardoso), the staff in Polymer Lab (James Hsiao), the Education Office (Linda Fuhrman and Gail DiDonato), and my supervisors who have led me through my masters thesis project (Joseph Charest and Sarah Tao). I also want to thank the other Draper Lab Fellows that have worked with me during my time here.

I would like to thank my MIT advisor Carol Livermore for taking time out of her busy schedule to provide guidance and suggestions. I would also like to thank the various MIT professors that have inspired me along the way.

Above all, I would like to thank my family. I would like to thank my father, mother, and sisters for their tremendous, unwavering support and love throughout this thesis, my Caltech undergraduate years, and life as a whole.

I apologize if I inadvertently left anyone out, but so many people were helpful and I am grateful for their kindness.

FUNDING ACKNOWLEDGMENT

Funding support from the Foundation Fighting Blindness is gratefully acknowledged.

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<thead>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>BioMEMS</td>
<td>Biological microelectromechanical systems</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DRIE</td>
<td>Deep Reactive Ion Etch</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered saline solution</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
</tr>
<tr>
<td>MEMS</td>
<td>Microelectromechanical systems</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(DL-lactide-co-glycolide)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>RIE</td>
<td>Reactive ion etch</td>
</tr>
<tr>
<td>RPC</td>
<td>Retinal Progenitor Cells</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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Chapter 1: Introduction

1.1 Regenerative Tissue Engineering

Regenerative tissue engineering is an emerging multidisciplinary area that utilizes a combination of cellular biology, medicine, and engineering to restore tissue and organ functions. The underlying principle behind this field is the discovery of methods to manipulate cells into performing specific roles. This requires careful understanding of how best to manipulate stem cells to differentiate into specific cells. Applications could then include culturing cells in vivo or in vitro, implanting them into organs, or developing tissues in vitro for drug testing. Several successful clinical trials for regeneration have already taken place for simple tissues such as skin and cartilage\(^1\). In the past two decades, accomplishments in the field of regenerative tissue engineering include characterization of stem cells under specific microenvironments, development of scaffolds and delivery vehicles, techniques to achieve viable engineered constructs using cells and biomaterials, and development of bioreactors to grow cells. Regenerative tissue engineering may be the solution to some of the most complex problems in medicine. While the human body can regenerate various body parts, such as skin and bone tissue, there are numerous other vital body organs that cannot be regenerated, such as the eyes and control nervous system. Diseases can also destroy the complete usage of specific organs that result in the loss of any regenerative capability. A strategy for tissue engineering can be used in treatment for these instances where regeneration is not foreseeable.

1.2 Cell Source: Stem Cells

Stem cells are living cells that can proliferate indefinitely and renew themselves at a high rate. Meanwhile, they continually maintain the potential to divide through mitosis or differentiate
into cells specific to a patient’s needs. The potential to differentiate into several types of specialized cells makes them attractive for study in tissue regeneration.

There are two types of stem cells: embryonic stem cells, which are isolated only from the inner cell mass of blastocysts, and adult stem cells (also called somatic stem cells) that are found in all organ tissues. Embryonic stem cells can potentially differentiate into all cell types in the body since they are pluripotent while adult stem cells are restricted to differentiating into various cell tissues from their originating organs. In addition, embryonic stem cells can be grown easily in culture, while adult stem cells are rare in mature organs such that isolating them from that particular tissue could be difficult. Adult stem cells have more potential as a viable solution because there is a smaller likelihood that they would be rejected by the body’s own immune system after transplantation[2]. Once adult stem cells start to differentiate (but before they are fully differentiated), they become progenitor cells. Progenitor cells are usually in the stage right between the adult stem cell and fully differentiated cell stages. They are multipotent in that they can differentiate into cells from multiple, but limited, number of lineages. Scientists have also developed methods to isolate and force specific differentiation among embryonic stem cells[3-5], as well as to turn adult human cells into induced pluripotent stem cells (iPSc) which behave exactly like embryonic stem cells[6, 7]. The advantages of using pluripotent stem cells versus progenitor cells are that stem cells can self-renew and replicate unlimited number of times while progenitor cells have limited number of self-renewal changes. The disadvantage is that progenitors are much easier to culture in vitro and in vivo than stem cells and more likely to differentiate into appropriate cell types.

1.3 Cell Delivery

Transplanting stem cells has primarily been performed through injection into tissues or organs, after which most cells have differentiated as expected[8-10]. However, these trials have also shown low cell survival rates and cell death after injection. Lack of direction for cells and
restriction of cell interactions after injections reduce the effectiveness of this transplant procedure. Thus, a reliable method for delivering cells into tissues and organs is needed. Research has shown that delivering cells on a biomaterial support structure, or scaffold improves cell survival rates and the ability to differentiate\textsuperscript{[11]}. The approach from this research is to design scaffolds that stem cells can be cultured on and then deliver both together to the site of degeneration. This approach would involve manipulating cell biology, chemistry, and morphology before transplantation and then using the scaffold to continue this manipulation \textit{in vivo} to induce desired cell responses.

1.4 Biomaterials

A biomaterial is anything that can be used for bio-related purposes and interacts with biological entities. In regenerative medicine, the biomaterials used directly impact the success of each scaffold implantation because of how sensitive cells are to their interactions to the substrate. Various materials, such as synthetic polymers, have been researched and tested for cell delivery potential. The ideal biomaterial used to fabricate scaffolds must be equipped to

(1) carry a large number of cells since an abundance of cells are needed for stem cell replacement therapies,

(2) deliver cells in the least intrusive way possible to not disturb the neighboring host environment,

(3) position cells close to each other to maintain cell-cell interactions and enhance cell survival rates,

(4) allow for physical and chemical cues to be included on substrate surfaces to instigate cell responses,
(5) maintain the precise stiffness/flexibility needed to attach correctly to tissues without compromising structural integrity (shape and form),

(6) be biodegradable in a specific time range to not impede the growth period of full tissue regeneration.

Additionally, the biomaterial must allow molding of 3-dimensional structures to better mirror tissues. Examples of specific biomaterials used in tissue engineering include poly(DL-lactide-co-glycolide) (PLGA) and polyglycerolsebacate (PGS) for capillary networks by stacking multiple layers together.

1.5 Cell-Substrate Interactions – Chemical and Biological Effects

In addition to the physical effect of scaffolds on cells mentioned above, chemical and biological factors of substrates also affect cell behaviors. Substrate surfaces may be altered chemically by coating or deposition of a top layer or by plasma treatment. These alteration methods are used to control protein adsorption, which affects cell adhesions, and to modify other important parameters such as wettability and surface charge to control cell morphology, migration, differentiation, and proliferation. For biological effects on cell behaviors, bioactive molecules such as adhesion ligands, growth factors, and enzymes may be put on the substrate surface to better mimic the extra cellular matrix and control cell behavior.

1.6 Cell-Substrate Interactions – Physical Topographical Effects

The connection between cells and their topographical environments strongly govern cell function. In past studies, cells have responded to two-dimensional synthetic topographic substrates with a wide range of reactions such as change in morphology, alignment, adhesion, differentiation, migration, proliferation, gene expression, and cytoskeleton organization. These reactions depend on the cell type, cue sizes, cue structures, patterning geometries (both
mechanical and chemical), scaffold materials, and stiffness\[16\]. Specifically, the responses of a wide variety of tissues to nanogratings, nanoposts, and nanopits have been studied because they are the physical features that are both representative of structures found in cell environment and relatively easy to fabricate on a micron level.

Cell alignment, attachment, adhesion, differentiation abilities and proliferation are some traits that have been researched in the past. For this particular project, the retinal cells that are the focus of this thesis will be tested for how well they align to gratings and ridge grooves while their differentiation potential will be tested for changes when seeded on post structures. These hypothesis come from similar reactions of other types of stem cells to ridge grooves (nano-gratings) and posts.

For example, in testing for alignment, various cell types such as fibroblasts, endothelial cells, stem cells, smooth muscle cells, and epithelial cells have seen increased alignment to the direction of gratings. Even neurites from cultured cells have shown extensions to align to gratings\[7\], while other types such as leukocytes, keratinocytes and monocytes have not shown distinct changes due to patterning\[7\].

Nano-gratings have also been shown to generally strengthen cell adhesion and lower proliferation rates while other features such as nanoposts and nanopits were shown to reduce cell attachment\[7\] while not revealing any discernable trend in proliferation\[7\].

As for differentiation, the role of submicron topography is still being explored as there have been mixed results so far. Only human mesenchymal stem cells reacted differently by differentiating into osteoblast lineage when cultured on nanopit substrates instead of smooth substrates\[7\]. No other types of cells had varying differentiation responses based on different substrate types.

In tissue engineering, these effects of cell alignment, attachment, adhesion, differentiation abilities and proliferation from topographical cues demonstrate the basics of how each individual sub-micron structure design affects cell behavior. The ultimate goal is to
eventually create the perfect sub-micron structure (or a combination of structures) that can instigate all desired effects (high differentiation potential, alignment, proliferation).

<table>
<thead>
<tr>
<th>Cells Used</th>
<th>Features</th>
<th>Materials</th>
<th>Cell Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoblast (primary and C2C12) [18]</td>
<td>Embossed ridge grooves (5um-75um)</td>
<td>Polystyrene</td>
<td>1). Increased Cell Alignment to Grooves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2). No impacts on cell density or differentiation</td>
</tr>
<tr>
<td>Human Neural Progenitor Cells [19]</td>
<td>Ridge Grooves (4um deep, 16um groove width)</td>
<td>Polystyrene films</td>
<td>1). Increased cell alignment to ridge grooves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2). No Impacts on cell differentiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2). High levels of photoreceptor markers detected in microwells</td>
</tr>
<tr>
<td>NIH 3T3 Fibroblasts [21]</td>
<td>Microchannels (2um wide, 10um deep seperated by 2um each)</td>
<td>Polymethylsiloxane (PDMS)</td>
<td>1). Cell morphology and structure oriented in compressive strain and channel directions</td>
</tr>
<tr>
<td>Human Corneal Epithelial Cells [22]</td>
<td>Ridge Grooves (4um deep, 16um groove width)</td>
<td>Tissue Culture Polystyrene Plates</td>
<td>1). Cell elongations and alignment perpendular to ridge groove direction</td>
</tr>
<tr>
<td>Fibroblast Cells [21]</td>
<td>Ridge grooves (lateral 2-10um and depths of 50-200nm)</td>
<td>Polymethylsiloxane (PDMS) substrate</td>
<td>1). Alignment to ridge groove</td>
</tr>
<tr>
<td>Endothelial Cells [17]</td>
<td>Ridge grooves (lateral 2-10um and depths of 50-200nm)</td>
<td>Polymethylsiloxane (PDMS) substrate</td>
<td>1). Alignment to ridge groove</td>
</tr>
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<td>Smooth Muscle Cells [21]</td>
<td>Ridge grooves (lateral 2-10um and depths of 50-200nm)</td>
<td>Polymethylsiloxane (PDMS) substrate</td>
<td>1). Alignment to ridge groove</td>
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<tr>
<td>Myoblast [18]</td>
<td>Ridge grooves (30um wide and 40um with spacing in between)</td>
<td>Polymethylsiloxane (PDMS) substrate</td>
<td>1). Alignment to uniaxial strain</td>
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<td>Human Endothelial Cells [21]</td>
<td>Ridge grooves (~600nm)</td>
<td>Polymethylsiloxane (PDMS)</td>
<td>1). Elongation and alignment along ridge grooves</td>
</tr>
<tr>
<td>Bovine endothelial cells [17]</td>
<td>Ridge grooves (2um)</td>
<td>Poly(glycerol sebacate)</td>
<td>1). Elongation and alignment along ridge grooves</td>
</tr>
<tr>
<td>Human Embryonic Stem Cells [17]</td>
<td>Ridge grooves (600nm)</td>
<td>Polymethylsiloxane (PDMS)</td>
<td>1). Elongation and alignment along ridge grooves</td>
</tr>
<tr>
<td>Human Mesenchymal Stem Cells [17]</td>
<td>Ridge grooves (350nm-10um)</td>
<td>Polymethylsiloxane (PDMS)</td>
<td>1). Elongation and alignment along ridge grooves</td>
</tr>
<tr>
<td>rC6 Glioma [17]</td>
<td>Ridge grooves (266nm)</td>
<td>Polystyrene</td>
<td>1). Elongation and alignment along ridge grooves</td>
</tr>
</tbody>
</table>

Figure 1-1: List of cell responses to specific physical topography. Articles which contain more in-depth information are cited.
The main physical topographies studied in research mentioned above were ridge-grooves and posts. Ridge-grooves seemed to induce cell alignment and cell elongation among most cell types. This change in stem cell elongation is important in cell therapy because cell elongation is one way in which cells become focused and specialized to perform a specific function. This differentiation process is very often signaled by cells changing shape so that they can better adjust to performing their new functions, and one way for spherical or flat-round cells to change shape is to elongate in a specific direction. For cell alignment, knowing ridge-grooves induce specific cell orientation reveals that physical topographical cues can confer cell organization *in vitro*. This insight would be important to retinal tissue engineering since tissues organization and arrangement of cells into layers or arrays is key for good visual processing and for function optimization in the retina.

1.7 Bio-Micro-Electro-Mechanical-Systems (BioMEMS)

The previous section discussed a myriad of evidence to support the effect of topographical cues on cell behavior. Some techniques that create these micro/nano structures include 3D printing, electrospinning (for producing polymer fibers as scaffolds), and bioreactors\(^{12}\). However, this project mainly focuses on the use of microfabrication. Fabricating these scaffold surfaces with sub-micron topographical cues requires knowledge and techniques developed early on in mechanical and electrical engineering.

Since 1961, microfabrication techniques have been used in the semi-conductor and microelectronics industry to create micro-electro-mechanical systems (MEMS)\(^{23}\). The microfabrication field involves techniques in creating minuscule structures down to the micron and nano scale levels, which are necessary for fabricating integrated circuits as well as for very-large-scale-integration (VLSI) technology. Microfabrication has a unique capacity to precisely control sub-micron architectures, a highly desirable tool in the regenerative tissue engineering field. These minuscule microarchitectures, in the range of cell sizes, can be used in
combination with the right biomaterial to create effective scaffolds for cell delivery. Moreover, the ability to fabricate large microstructured areas for high throughput technology makes this technique even more desirable for research on micro-electro-mechanical systems specifically for biomedical applications (bioMEMS).

Microfabrication techniques like photolithography can also allow for even smaller nano-scale features to be fabricated over large areas. Photolithography is a fabrication technique which requires applying a coat of photoresist onto silicon wafer substrates followed by exposure to ultra-violet light through a patterned mask to soften or harden specific patterns in the photoresist. Photoresist is a photosensitive polymer film that can shield the underlying wafer from being etched in an etch process. It can also be selectively patterned when bombarded with ultraviolet light so that the patterned areas are either more or less easily removed by a developing solution. The more readily removed photoresist then dissolves away in developer solution, leaving the patterned area exposed for etching in the deep reactive ion etcher. Light diffraction wavelength sets the minimum size for which this method can be used, but electron beam lithography can overcome this obstacle\textsuperscript{[15]} and enable patterning of smaller features. The submicron physical topographical cues created in this way directly influence cellular responses in alignment, morphology, and differentiation. Soft lithography (or imprint lithography) techniques, which involves using silicon wafers to imprint patterns or mold replicas onto a biomaterial substrate like polydimethylsiloxane (PDMS), allow for replication of low-scale structures at a cheap and fast rate while retaining the resolution of each structure\textsuperscript{[24]}. In turn, these PDMS molds can be used to replicate structures onto scaffolds.

1.8 2D vs. 3D Scaffolds

Regenerative tissue engineering aims at developing sophisticated synthetic scaffolds to mimic the cell-tissue environment as much as possible, which includes surrounding a cultured cell with other cells and also an extracellular matrix (ECM). However, one feature that many
researchers recently started to look at is the dimensionality of scaffolds. Two dimensional (2D) scaffolds do not accurately depict the micro-environment of an organ or tissue, in which all cells differentiate, grow, migrate, and respond to three dimensional (3D) environments. Furthermore, 2D substrates allow for cell migration out of the scaffold, and cell adhesion becomes problematic in transplantation due to the shearing of cells off of the scaffold.

Scaffold dimensionality has also been seen to affect cell polarity (direction of front and back). Cells in a 3D environment display normal cell polarity, but cells in a 2D environment do not\textsuperscript{[16, 25]}. Cells in a 2D plane feel a different sub-micron environment on one side (if any) than the other, while cells on 3D scaffolds can feel the same sub-micron environment on all sides. Similar to being in ECM, cells also expose more body surface area to the surrounding environment for attachment or adhesion in 3D scaffolds and increase cell-to-cell\textsuperscript{[16, 25]}. In addition, by providing more precise structural environments and improving cell position restraints, 3D scaffolds can also provide more bio and chemical cues\textsuperscript{[26]}. Past experiments using fibroblasts have shown that cells cultured in 3D scaffolds have increased cell adhesion, migration, shape solidification, and proliferation than those cultured on 2D substrates\textsuperscript{[15]}. 
Chapter 2: Retinal Project

Background

2.1 Retina Anatomy

The retina, located in the back inner surface of the eye, is about 0.2mm thick, with a diameter of approximately 42 mm\textsuperscript{[27]}. It contains multiple layers of light-sensitive tissue that send chemical and electrical impulses to the optic nerves by utilizing photoreceptors. Photoreceptors are a specialized type of neuron nerve cells, which capture and convert light into electrical signals to the retina. The photoreceptors create this effect by absorbing photons from the light coming into the retina and changing their membrane potentials. There are two main types of photoreceptors in the retina: 75 million rods and 7 million cones. The rods, located in the periphery of the retina, are responsible for central vision. The cones, located in the center of the retina, are responsible for night vision. The retina also contains ganglion cells, which grow dendrites and have long axons attached to several areas of the brain and serve as the main connector of impulse to the brain. The back of the retina faces the retinal pigment epithelium, which is a layer of cells that provides nutrients for the various retinal cells.
Figure 2-1: Schematic drawing of the (human) retina including an enlarged section of the retina (Reproduced with the written permission of Helga Kolb, Professor Emeritus at Moran Eye Center)

Figure 2-2: Enlarged view of the retinal layer from previous picture (Reproduced with the written permission of Helga Kolb, Professor Emeritus at Moran Eye Center)
2.2 Retina Diseases

Retinal diseases specifically affect the retina and can affect the macula (yellow oval shaped spot near the retina center that absorbs excess blue and UV light) or the fovea (center of the macula responsible for sharp central vision). While many retinal diseases cause common symptoms and treatments, each disease has its own unique attributes. Common retinal diseases and defects include retinal detachment, diabetic retinopathy, epiretinal membrane retinal tear, and macular hole. Two untreatable retinal diseases that eventually lead to blindness but can potentially be cured by regenerative therapies are Age-Related Macular Degeneration (AMD) and Retinitis Pigmentosa (RP).

2.2.1 Age Macular Degeneration

Age-Related Macular Degeneration is the main cause of vision loss in the United States for people above the age of 65. Currently, about 2 million people suffer from this disease any given year. There are two types of AMD: a dry form and a wet form. In the dry form, small
deposits, called drusen, accumulate between the retina and the choroids (the vascular layer of the eye that contains connective tissue\textsuperscript{[27]} and cause gradual degeneration of retinal layers. In the wet form, blood vessels grow and expand under the retina, causing blood and fluid to leak, which ultimately kill the photoreceptor cells needed for light sensitivity. In both forms, the loss of these photoreceptors ultimately causes blindness among victims.

![Normal View, Age Related Macular Degeneration, Retinitis Pigmentosa](image)

Figure 2-4: The left shows the view of someone with healthy retina. The middle shows the view of someone with AMD. The right shows the view of someone with RP. Photographs are not copyrighted and maybe reproduced without permission. Images courtesy of The National Eye Institute (NEI)

### 2.2.2 Retinitis Pigmentosa

Retinitis Pigmentosa (RP) is a genetic eye disorder that also ultimately leads to blindness. It consists of retinal dystrophies and degenerative disorder in the retinal pigment epithelium (RPE) caused by anomaly in more than 100 different genes\textsuperscript{[28]}. These mutated genes send out incorrect information to photoreceptor cells which lead them to either make the wrong protein or the wrong amount of a protein (when cells need the exact dose of a particular protein to perform adequately). One form of RP is cone-rod dystrophy, where the anomaly affects the RPE and cone photoreceptors. RP is more common in the form of rod-cone dystrophy, where cell death occurs in rod photoreceptors. The rod outer segments slowly shorten and the rod photoreceptor loss causes loss of peripheral vision and night vision\textsuperscript{[28]}. Patients usually experience initial symptoms of this night blindness and tunnel vision before finally reaching blindness.
Because both of these diseases (AMD and RP) cause the loss of photoreceptor cells that do not regenerate themselves, and because there are no existing drug therapies, regenerative medicine holds much potential for curing these diseases.

2.3 Thesis Project Overall Goal

The overall goal of this thesis project is to investigate a method of developing implantable scaffolds for use in retinal tissue engineering. While research has gone into methods of rescuing damaged retinal tissues, there is still no proven visually beneficial treatment for retinal diseases such as AMD and RP. Because retina damages in most cases are irreversible, one strategy is to use cell-based therapy to preserve and restore vision to normal conditions\cite{29-31}. Past research has already shown that transplanted cells may be able to replace dying photoreceptors of the host retina using bolus injections\cite{20,32,33}. These bolus injections of cell suspensions into the subretinal space revealed enough proof of principle for stem cell integration, but such a method also had drawbacks that will not satisfy future clinical requirements\cite{29,32,33}. Although there were stem cell integrations into a retinal layer as photoreceptor cells, bolus injections also led to residual cells in the subretinal space that did not integrate well\cite{20,32,33}. The prolonged presence of these cells might increase the risk of retinal
gliosis and other prolonged retinal detachment problems later on. Additionally, the lack of guidance cues from bolus injections also failed to yield high cell survival rates, photoreceptor morphologies, photoreceptor chemical marker expressions, or proper organizations. Thus, in scenarios where the original retinal cell population has been diminished and where the local retinal cytoarchitecture has been obstructed, other mechanism will be needed to bestow organization to grafted cells. Later research following bolus injections found that delivering cells with fabricated scaffolds had enhanced the survival of cells as well as their ability to differentiate.

A biomaterial scaffold could advance the arrangement of grafted cells on layers that are seen in the normal retina. With enough adhesion to the surface, culturing cells on a polymer scaffold leads to an implant that exhibit inherent structural organization that bolus injections did not provide. A biomaterial scaffold may better simulate native guidance cues, which in turn, influences cell responses. In fact, results from last chapter that showed cell morphological changes from microenvironment was implying that such physical cues could induce cell differentiation in vitro. This thesis project aims to test for similar results involving retinal stem cells in an attempt to understand how best to control these retinal stem cells when they are implanted in future projects. Therefore, how physical microenvironment affects retinal stem cell differentiation will be closely studied in thesis experiments. In addition to cell differentiation potential aspect, because tissues organization and arrangement of cells into layers or arrays is key for good visual processing and for function optimization in the retina, how much each individual cue type affects cell organization must also be well understood to establish well-rounded model representative of the retina. Therefore, while how physical microenvironment affects differentiation will be looked into, how these physical cues affect cell organization and arrangement will also be looked into as well.

2.3 Use of Retinal Progenitor Cells in Retinal Tissue Engineering
There are different ways of getting photoreceptor cells. One option is retinal progenitor cells (RPCs), which are stem cells that have been successfully isolated and derived from neural retinal tissue. These cells have been successfully isolated from embryonic and adult mice in addition to human neuro-retinal tissues. They are regenerative cells that can differentiate into other different retinal cell types, including rod and cone photoreceptor cells. They have also displayed the capability to be replacement cells for photoreceptors and can integrate into the retinal layers [34-40]. Recent studies on smooth microfabricated scaffolds demonstrated increased cell attachment, organization and higher gene expression of RPCs for photoreceptor markers recoverin and rhodopsin [33], making it possible to differentiate RPCs towards photoreceptor-committed cells in vitro prior to transplantation.

2.4 Biomaterial in Retinal Tissue Engineering

Biomaterials used as scaffolds for retinal transplantation must meet several main criteria. They must be thin enough for subretinal transplantation (< 10μm), deliver a large population of stem or progenitor cells, be stiff enough for surgical transplantation, be flexible enough to curve around the back of the retina, and be biodegradable in the eye.

Many factors must be determined precisely when deciding on material such as: film thickness, mechanical properties, solvent used if material needs to be dissolved first, concentration of polymer to use, controllability of surface substrate (uniformity), spin coating speed, and heating process parameters.

One of the most recent practices in tissue engineering and regenerative medicine is to use degradable polymers. They can be absorbed by the host once implanted such that cells cultured on them can function freely. However, one concern is the high toxicity levels of the released monomers of the biomaterial once it degrades [41]. Because of this, Poly(ε-caprolactone)(PCL) degradation has been examined. The PCL first starts to degrade in vivo by decreasing in molecular weight without deformation of the material for about 2 years and then
gradually breaks into pieces that will eventually be excreted from the body\cite{42}. No traces of this biomaterial are detected after the excretion. These findings make PCL favorable to use for this tissue-scaffold implant experiment since the imprinted form will at least hold for 2 years.

PCL is also selected based on looking at previous studies comparing its characteristics to other similar tissue culture polymers. PCL has a low melting temperature, and is also soluble in many different solvents\cite{41,43-46}. It has a thermoplastic property along with a high molecular weight that makes it easy to use for spinning thin films\cite{41,43-46}. It crystallizes well at room temperature, which makes it convenient to develop.

Other than having low degradation rate and biocompatibility, past experiments have also shown other advantages of using PCL films. PLGA, poly(DL-lactide-co-ε-caprolactone) (DLPLCL), and PCL were all tested for nanostructure formation, and PCL was able to produce the best quality of nanostructures\cite{41,43-46}. Past cell culture experiments have shown cells such as fibroblasts grew well on all different types of surfaces regardless of solvent used with PCL\cite{41,43-46}. Because of all the explanations above, PCL was ultimately chosen as the biomaterial to use for scaffold in this project. Additionally, the flexibility/curvature the substrate must achieve in this project implies that thin film structure is needed.

### 2.5 Thesis Objectives

This thesis project takes the entire cell-therapy techniques and discussion above and applies it to regenerative therapy for the retina. The basic hypothesis for this project, from looking at past research results discussed in Chapter 1, is that PCL scaffolds, scaffolds with the selected biomaterial from last section, with embedded sub-micron topographical cues ridge grooves and posts (physical cues from past research) will induce stronger cell alignment, higher elongation and higher levels of differentiation marker expressions on RPCs than PCL scaffolds with no physical cues on surface.
To test this hypothesis, this research aims to develop PCL substrates with smooth surfaces, surfaces with ridge-grooves, and surfaces with posts. The PCL substrates are 5 microns thick such that when cells are seeded on the substrate, there is enough room in the retinal layers for the substrate with the seeded cells to be implanted. Retinal progenitor cells will then be cultured on this substrate to test for differentiation levels and for morphological changes. Overall, the work in this thesis has the following goals:

1). **To develop a method to produce desired sub-micron environments on PCL.** Past research has shown that micro-fabrication techniques can create structures in the sub-micron scale. In this part of the research, a method to microfabricate desired patterns and transfer them onto PCL thin films is developed. To accomplish this, we research specific sub-micron cues that have the best probability of instigating desirable cell responses in RPCs and design these sub-micron cues such that they can be microfabricated on silicon wafers and accurately be transferred into PCL. A novel templating process is developed to imprint sub-micron cues into biodegradable (PCL) thin films between 5-10μm thick with minimal deformation to the imprinted features and to optimize the appropriate parameters for manufacturing of PCL films by performing quality analysis of the films.

2). **To investigate the physical microenvironment cues that instigate desired reactions from RPC.** Past research has shown that certain specific physical shapes such as sub-micron ridge grooves, needles, posts, and squares can change the morphology of cells. In this aim, the cell response to their microenvironment will be examined and the physical and chemical changes that occur in these cells such as the elongation, alignment, and expression of photoreceptor markers will be quantified.
2.6 Detailed Hypothesis

The detailed hypothesis for this research was that the proposed method of microfabrication could create sub-micron structures with high accuracy and that these features could be replicated onto thin PCL films without losing resolution. Achieving these goals would allow us to establish a set method of creating a high throughput of arrays in less time and cost than we otherwise would need with 3D-printing or electrospinning.

As with cell culture study, we expect physical cues such as ridge grooves and posts would cause both physical and chemical changes on RPCs. Specifically, ridge grooves would cause elongation among cells and align them parallel to the direction of the ridge grooves in such a way that we can establish a known method of molding RPCs into photoreceptor cell shapes. As for differentiation potential, we expect the physical cues and the biomaterial to instigate cells’ differentiations even in the absence of biochemical signaling. Accomplishing this result will verify that the technique to culture and differentiate RPCs on scaffolds first and then
implant them together into the degeneration area of the retina is possible and that this method can potentially replace bolus injection into the retina.
Chapter 3: Initial Trial with 661W Cells

This chapter describes experiments to show proof of concept and to do trial testing on easier-to-culture retinal cell lines before implementing the method discussed in this chapter on real retinal progenitor cells. The experiments included the characterization of suitable materials for the biomaterial substrates, developing silicon master mold with correct configuration of physical topography, developing substrates off of the master mold with the right thickness and the right biomaterial, culturing retinal cell line on thin films, staining them to test for level of photoreceptor marker expression, and analyzing SEM images in quantifying morphological changes.

3.1 Poly(ε-caprolactone) Film Characterization Study

Developing high quality PCL films is important for this experimentation process. Good film and feature quality lead to easier future cell deposition as well as more accurate results. Thus, testing for optimal parameters such as concentration, spin rate, and baking or molding temperature is necessary.

For concentration, we looked at previous research to see how the thickness of the film correlates to the concentration of PCL for different spin speeds[^44]. From this, we determined that for films of about 5μm or thinner, we want to use a concentration of around 0.1g/mL if we want to keep the spinning rate lower than 2000rpm.

Once 0.1g/mL concentration of polycaprolactone was decided, we tested for different spin rates of PCL solution. A standard spin coating procedure was followed to spin and coat PCL solutions on silicon wafers at different rates for a fixed spinning time under constant room
temperature. The film was then cut at several sections and the thickness was measured at a Tencor (Alpha-Step IQ Surface Profiler) machine.

The resulting thickness vs. spin speed is below:

<table>
<thead>
<tr>
<th>Spin Speed (RPM)</th>
<th>Average Film Thickness</th>
<th>Standard Deviation (sample size = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>11.86</td>
<td>1.57</td>
</tr>
<tr>
<td>750</td>
<td>10.56</td>
<td>1.21</td>
</tr>
<tr>
<td>1000</td>
<td>7.28</td>
<td>0.52</td>
</tr>
<tr>
<td>1500</td>
<td>4.88</td>
<td>0.1</td>
</tr>
</tbody>
</table>

![Figure 3-1: PCL film thickness dependence on spin speed](image_url)
From the chart above, spinning at 1500rpm yielded the results that we want in lowering the film thickness to less than 10μm.

In obtaining the optimal baking/molding temperature in the oven, several trials were done at different temperatures to test the quality of films:
From looking at several sets of SEM pictures of PCL molded at different temperatures, the film with the 70°C baking period turned out to have the highest film surface quality because it accurately replicated the mold. Thus, we established our film-developing method to be 1g/mL at 1500rpm for 30s with baking/molding at a temperature of 70°C for 15 mins.

3.2 Initial Testing of Sub-Micron Features

From literature reviews discussed in Chapter 2, ridge grooves and posts were chosen as the main two features to be tested with our original retinal cell line (661W). For this chapter, only cells on ridge grooves will be analyzed while cells on both ridge grooves and posts will be analyzed in the next chapter.

3.2.1 661W Cells

661W is a photoreceptor cell line cloned from retinal tumors that was arising in genetically modified mice expressing simian virus (SV) 40 T antigen (Tag) under control of human interphotoreceptor retinoid binding protein (IRBP) promoter\(^{[47]}\). Cellular analyses have shown these cells only embody cone photoreceptor markers and not rod photoreceptors, which indicate that these cells are from a cone photoreceptor cell line and should give insights to how RPCs might behave once it differentiate into cone photoreceptor cells. (661W Cell Line was from Al-Ubaidi Laboratory at University of Oklahoma Health Sciences Center.)
Original testing of retinal cell response on sub-micron topography used 661W cells because of the relative simplicity in culturing them and because studies have shown that 661W cells displayed retinal progenitor cells characteristics[^48].

### 3.2.2 661W Cells Protocols

500mL of DMEM (Invitrogen, 1196-065) was mixed with supplemental aliquots of putrescine (Sigma, P-7505), hydrocortisone 21-hemisuccinate (Sigma, H-2270), progesterone (Sigma, H-2270), 2-mercaptoethanol (Sigma, M-6250) and antibiotic-antimycotic (Invitrogen, 15240-062) to create growth medium for 661W cells. Medium was also filter-sterilized into a sterile bottle before being used on cells.

661W cells were cultured in an incubator environment at 37°C, and growth medium was changed biweekly. Cells were passaged once they hit about 90% confluency as indicated by brightfield microscope inspection. 0.025% trypsin-EDTA was used as agent to remove unnecessary cells from the sterile bottle. Cells were split at a ratio of 1:5 into new bottles. For freezing cells, regular growth medium was mixed with 10% DMSO before freezing in a -20°C fridge.

### 3.3 Thin Film Method and Materials

#### 3.3.1 Mask Design

The mask layout for this specific chrome mask was designed to include a field of multiple small squares of features such that a high throughput testing process was possible on various designs. Features such as 5:1μm ridge grooves, checkerboards, square posts, and grids were included in this mask. The picture below shows the type and size of patterns (i.e. 05 = 0.50μm, 10 = 1.0μm). All features have height of 1.0μm.
3.3.2 MEMS Fabrication Procedures

Fabrication techniques were used to create the submicron topography on wafers. Blank silicon wafers with a layer of 1µm thick oxide were submerged in 3:1 piranha mixture for cleansing to remove any nano-particles on the surface. Wafers were then rinsed in de-ionized water to wash off any chemical residue and then dehydrated for 30mins at 110°C to evaporate all water as preparation before spinning on Hexamethyldisilazane (HMDS) and photoresists since photoresists are hydrophobic and will not stick to wafers well unless they are completely dry. HMDS was spun on the wafers (5000rpm for 10s at acceleration of 1000rpm/s) to create a thin layer to bolster the adhesion of the photoresist. Wafers were then hotplate-baked for 1 minute at 115°C. Photoresist S1805 (Shipley, Product Code: 41200) was spun on at a spin speed of 3500rpm for 20s. S1805 was chosen for its layer thickness of 5000Å. Spin-coating was used since it was the only efficient way to coat the wafer uniformly. The coated wafers
were then prebaked to dry out the photoresist and crosslink the photoresist to transform it from liquid to solid form.

Wafers were then exposed on MA-6 machine to ultra violet light through a chrome mask for around 4.5s. UV light can resolve smaller features than visible light. Different time ranges were tested for the best exposure time that yielded the best resolution. Afterwards, wafers were developed in MF-319 developer solution (Rohm HAAS Electronics Material) to rinse away exposed photoresist, exposing parts of the wafers for etching. Wafers with hardened photoresist were put into a Deep Reactive Ion Etcher (DRIE) for etching of 1um. Passivation at 100 square cubic centimeters per minute flow was done with octafluorobutene (C₄F₆) while etching was done with sulfur hexafluoride (SF₆) gas at 130sccm and oxygen gas at 13sccm for 8m11s. Etched wafers were then finally checked in the Tencor (Alpha-Step IQ Surface Profiler) for depth and submerged back in piranha for final cleaning.

![Schematic of photolithography process](image)

**Figure 3-6: Schematic of photolithography process**

### 3.3.3 Thin film Procedures

Once fabrication of sub-micron structures in the silicon oxide master-mold was finished, thin films could start to be developed off the patterns. 0.1g/mL concentration PCL solution was prepared by dissolving polycaprolactone pellets (Molecular Weight = 80,000) (Sigma-Aldrich, 440744-500G) in dichloromethane(Sigma-Aldrich, D65100-2L) and stirring them for 3 hours. The silicon master mold was then submerged into 2% microsoap solution for 5 minutes to create a sacrificial layer on the master mold surface. The wafer was spun at 1500rpm to spin off the remaining residue/particles to prepare it for film deposition. 10mL of the 0.1g/mL PCL was deposited on silicon master mold uniformly and then spun again at 1500 rpm for even
distribution and for the solvent to be evaporated at room temperature. The wafer with the PCL thin film was put in a 70°C oven for 15 minutes to mold PCL around the sub-micron topography followed by submersion in deionized water for thin film lift off (deionized water allowed the PCL layer to be peeled from the surface). Once the films were dried, they were treated with oxygen plasma (200W, 270mTorr, ENI Model N: ACT-3B) for 30s to increase hydrophobicity for cell adhesion during cell culture.

3.4 661W Cells Staining
Once films were developed, they were mounted on clear plastic coverslips and treated under ultra violet light for 30mins in hood. Films mounted on coverslips were then treated in 1mL 70% ethanol for 30mins before depositing a pre-set concentration of cells. Films and cells were then rinsed with 2ml phosphate-buffered saline (PBS) five times for 5 minutes each before finally being replaced with 2mL of 661W growth medium and placed in an incubator at 37°C.

Cells were grown for set time periods of 3, 5, and 7 days on film. After the time period, cells were fixed in 3.7% formaldehyde for 15mins at room temperature and rinsed 3 times after fixation. Cells were then permeabilized in 0.1% triton-100 for 15mins.

Cells were then immunolabeled with primary antibody Opsin, a colorless protein that illuminates when combined with retinal protein, (200μL for each coverslip) and incubated at room temperature for 60mins before being rinsed with PBS. Cells were then incubated with the conjugate (568 rabbit anti-goat for staining of other cell structures) for 60mins at room temperature for 37°C followed for PBS rinse. Samples were lowered facedown onto anti-fade DAPI reagent and left to cure in a dark room for 24hrs before the sample edge was sealed with nail polish.

3.5 Preparations for SEM

While half the samples were used for staining images, the other half were fixed for SEM images. Primary fixative of 3% Glutaraldehyde in Sucrose-Cacodylate buffer (1.5mL of 50% Glutaraldehyde in 25mL) was prepared along with Sucrose-Cacodylate buffer containing 0.1M sodium cacodylate and 0.1M sucrose (2.14g sodium cacodylate and 3.424g sucrose in 1L sucrose-cacodylate buffer).

Fixed cell samples were placed with sucrose-cacodylated buffer and left for 5 minutes. Buffer was then removed, and the rinse procedure was repeated for an additional 5 minutes. Cell samples were then dehydrated by adding and replacing solutions of ethanol in a graded
series of concentrations of 35% for 10 minutes, 50% for 10 mins, 70% for 10mins, 95% for 10 mins and 100% for 10 mins.

Samples were then sputter-coated using a Cressington Sputter Coater for 45s to deposit a layer of gold particles on the cell samples before inserting them into the SEM (Hitachi S-3500N).

3.6 Statistical Analysis

Statistical analysis was done using unpaired Student’s t-tests with varying variances. Two-tailed tests were elected, using $\alpha = 0.05$ with mean differences considered statistically significant at $P<0.05$.

3.7 Results

3.7.1 Microfabrication Accuracy

Micropatterned silicon wafers were fabricated to contain 1µm-to-5µm ridge grooves, and 0.50µm diameter posts using photolithography techniques and deep reactive ion etching. After the etching and piranha clean, the designed 0.50µm diameter posts and 1µm-to-5µm ridge-grooves had (in µm) average dimensions of 0.51 ± 0.05, 1.05 ± 0.10, and 5.28 ± .12, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Ridge(µm)</th>
<th>Groove(µm)</th>
<th>Posts(µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wafer</td>
<td>1.05 ± 0.10</td>
<td>5.28 ± 0.12</td>
<td>0.51 ± 0.05</td>
</tr>
</tbody>
</table>
3.7.2 Thin Film Accuracy

Films peeled from smooth blank wafers were used as controls to compare the effects of physical cues. Film thickness depended on spin speed, and in this case, it was 1500 rpm for 5μm thickness. As for quality of topographical cues, the designed 0.50μm diameter posts and 1μm-to-5μm ridge grooves had (in μm) average dimensions of 0.54 ± 0.07, 1.06 ± 0.12, and 4.90 ± .25, respectively.
<table>
<thead>
<tr>
<th></th>
<th>Ridge (µm)</th>
<th>Groove (µm)</th>
<th>Posts (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wafer</td>
<td>1.06 ± 0.12</td>
<td>4.90 ± 0.25</td>
<td>0.54 ± 0.07</td>
</tr>
</tbody>
</table>

Thin Film Characterization

![Bar chart showing width of ridge, groove, and posts features with error bars.]

Figure 3-10: Characterization of physical features in thin films

![Image of overall film and SEM images of ridge-grooves portion of film with measurement labels.]

Figure 3-11: (A) Image of overall film (B,C,D) SEM images of ridge-grooves portion of film with measurement labels
3.7.3 Morphology Changes

![Figure 3-12: (A) 661W cells on posts (B) 661W cells on 1:5 ridge-grooves](image)

Scanning electron microscopy images were taken at pre-determined positions. These images were then analyzed using ImageJ for their elongations and alignments. For elongation, the outline of each individual cell was traced and a best-fit ellipse was automatically fitted to that trace. The major axis and minor axis was then measured. The elongation was then calculated using the size of the major and minor axes:

\[
\text{Elongation} = \frac{\text{Major Axis} - \text{Minor Axis}}{\text{Major Axis} + \text{Minor Axis}},
\]

where \(E = 0\) indicates the cell is perfectly circular and \(E = 1\) indicates perfect cell elongation.

For measuring alignments, the direction of the major axis was measured relative to the direction of the ridge groove. For smooth PCL surfaces and PCL surfaces with posts, the angles were measured between the horizontal x-axis of the film (direction of where ridge-grooves would be) and the major axis of the cell best fit ellipse.
Figure 3-13: Best fitted ellipse of 661W cell on (A) ridge-grooves film and (B) smooth blank film

Figure 3-14: Outline of alignment angle measurement of cells on ridge-grooves

Figure 3-15: Box and whisker plot of 661W cell elongation between cells on ridge-grooves and cells on blank control film. Lower and upper box edges correspond to 1st and 3rd quartile, respectively while the middle red line corresponds to median (the same type of box and whisker plot will be applied throughout this thesis)
Figure 3-16: Average of 661W cell elongation between cells on ridge-grooves (0.53) and cells on blank control film (0.10)

<table>
<thead>
<tr>
<th>Elongation of Control Vs. Ridge Groove</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
</tr>
<tr>
<td>Significance</td>
</tr>
</tbody>
</table>

Figure 3-17: P-value of 0.0002 shows there is a statistically significant difference between elongation of 661W cells on ridge grooves and those on control smooth film.

Figure 3-18: Box and whisker plot of 661W cell alignment angles on ridge-grooves and smooth blank films. Ridge-grooves alignment angles are defined between cell’s major axis and ridge-grooves while smooth alignment angles are defined as cell’s major axis and x-axis of samples. By this convention, having lower alignment angles mean being closer to having perfect alignment.
Our results suggest that sub-micron topographical features can induce specific cell orientation/alignment and morphological changes. The alignment angle, defined as the angle between the vector of cell elongation direction and predetermined direction of topography, varied depending on the properties of the thin film. About 400 cells from each type (patterned and smooth) of film were studied. For patterned thin films with ridge grooves, 52% were oriented 10 degrees or lower from perfect alignment ($\theta = 0$) with 76% of cells within 20 degrees or lower. The population of the lower angular ranges is much higher than the 11% that would be anticipated in each 10 degree range for a random distribution across angles, indicating that the samples with ridge grooves strongly promote alignment of the 661W cells. For smooth flat thin films, the distribution was much more random, with an average of 16.5% of cells plus or minus a standard deviation of 6% aligning within each 10 degree window from 20 degrees to 80 degrees. The bar chart above also demonstrated most cells on control blank films did not have inclination towards any specific angle range since the distribution of those cells across most

Figure 3-19: Quantification of alignment angles of 661W cells on ridge-grooves film and smooth blank films. (note: Perfect alignment on smooth surface would be defined as cells to align in the horizontal direction of the film) Cells on smooth control 661W cells skewed more towards perfect alignment when on ridge-grooves films than on control films. Cells on control films also had mostly even distribution of cells orienting towards different directions. There were no cells that orientated within 20° of the ridge-groove direction or perpendicular to that direction, which attributes to an unknown reason.
angles had very little variation. It is surprising that 0% were found to be oriented less than 20 degrees or greater than 80 degrees from perfect alignment on the control sample. If the distribution were truly random in the absence of contact guidance from features like the ridge-grooves on the patterned films, the distribution would be even across all angles. The absence of cells in the angular ranges near 0 and 90 degrees is attributed to an unknown reason. However, for the angle ranges that had cell oriented towards it, the even distribution on the control samples can be proven by looking at the standard deviation of percentage of cells that align in any particular range:

![Standard Deviation in % of cells aligning towards any particular angle](image)

**Figure 3-20: Standard Deviation of cells aligning towards any 10° range**

The above chart shows that for smooth control films, the percentages of cells aligning to any particular degrees are similar. This translates to that no cells prefer alignment to a particular angle over all other angles.

As for the average alignment after 7 days of culture, the cells patterned thin films had statistically significantly lower angles away from perfect alignment (average of 50° for smooth film vs. 12° for ridge groove; $\alpha = .05$, $P < .001$), indicating that the ridge groove patterns had strongly influenced the orientation of 661W cells used on thin films.
As for elongation, cells cultured on the ridge groove thin films showed a statistically significant increase in elongation compared to those cultured on the smooth thin films ($E=0.54$ for ridge grooves compared to $E=0.10$ on average for control films; $\alpha = .05$, $P < .001$).

### 3.7.4 Cell Staining (Immunocytochemistry Process)

![Figure 3-21: (A) Stained Nucleus on ridge-grooves film (B) Opsin stain on ridge-grooves film (C) Stained for cell body on ridge-grooves (D) Cell nucleus(in blue) on control film](image)

Staining procedures were used to verify elongation and alignment of cells to ridge groove. Staining for cell nuclei using DAPI verified cell structure outline while staining for Opsin, rod photoreceptor cells marker, showed low levels of photoreceptor cell differentiation. While none of these procedures were analyzed quantitatively, they demonstrated the procedures needed for staining in the next round and also verified the results analyzed through SEM images.
3.7.5 Discussion

The results shown in this section verified the ability of micro-fabrication to create sub-micron structures down in PCL down to 500nm-1μm with high accuracy and high resolution. The results have also shown that 661W cells interacted with the surrounding substrate in aligning more to ridge grooves as well as elongating parallel to them. This showed promise as to how much influence sub-micron topographies will have on retinal progenitor cells. Overall, this experiment with 661W cells served as a quick, useful tool for assessing what topographical features to aim for in the next experiment and what set of cell markers to specifically look for to determine cell differentiation and organization.
Chapter 4: Retinal Progenitor Cells Round 2

4.1 Introduction

4.1.1 Retinal Progenitor Cells

In addition to choosing the scaffold materials and submicron environment, the cell type used in culturing must be chosen to be able to integrate into the host environment and differentiate into photoreceptor cells. Past research has shown that already differentiated cells were not compatible with host retinal tissue\textsuperscript{[29]}, and that neural progenitor cells do not reach the final differentiation stage of the specific retinal cells needed\textsuperscript{[46]}. These past experiments led to the need to use retinal progenitor cells. Retinal progenitor cells used in this thesis project were obtained from mice. Eyes were removed and put in Hank’s Balance Salt Solution (HBSS, Invitrogen, Carlsbad, CA, USA), and neural retinas were dissected away from the optic disc and ciliary marginal zone. The retinal tissue was then minced with 0.1\% type 1 collagenase for 20 minutes at room temperature, and freed cells were collected through a mesh strainer (BD, Franklin Lakes, NJ, USA) followed by being centrifuged at 1,000rpm for 5 mins and re-suspended in neurobasal medium (NB; Invitrogen). Cells were then transferred to a 6-well plate and incubated at 37°C in 5\% CO\textsubscript{2} incubator. Maintaining these cells included adding 0.5ml of fresh medium 2-3 times a week and passaging cells once every 2 weeks.
4.1.2 Experiment Overview

The basic overview of this experiment is to fabricate a silicon wafer with ridge-grooves, posts, and combined ridge-grooves with posts. Ridge grooves were demonstrated in previous chapter to induce elongation and alignment. Posts were not analyzed in the previous chapter, but they have been shown to enhance differentiation potential of other types of stem cells in chapter 1. For this experiment, posts will be set up such that they are in hexagonal arrays since photoreceptors have been researched to be in arrays of hexagons in the eye\textsuperscript{[50]}. A patterned surface of combined ridge-grooves and posts has never been tried before, but we want to test whether the combination of both patterns would induce all the desired effects of ridge grooves and posts as individual patterns (alignment, elongation, and higher differentiation effects). Thus, the hypothesis for this experiment is that ridge grooves will induce elongation and alignment of RPCs and posts will induce higher levels of photoreceptor markers in RPCs while combined ridge-grooves and posts would induce all three effects.

This experiment will be accomplished by developing films off of the patterned silicon wafer master mold, seeding cells on for 7 days (since that is a normal period in which any effects on cells can be seen clearly), and fixing half the samples for SEM images and analysis and the other half for immunocytochemistry process. For the SEM images, cell elongation, alignment, area, perimeters, and processes will be analyzed. Elongation and alignment are analyzed for same reasons as previous chapters while cell areas, perimeters, and processes are measured because any significant changes induced by physical cues would show how cells
were reorganized and given structures. For immunocytochemistry (staining), CRX, Rhodopsin, and Recoverin will be analyzed since these three photoreceptor markers can represent differentiations of RPCs into photoreceptor cells.

4.2 Method and Materials

4.2.1 Mask Design Layout and Manufacturing

From our past experiment in the previous chapter, it was evident that ridge grooves instigated alignment and elongation of 661W cells, and the next step was to see if the same applied to RPCs. In doing this, precisely controlled ridge grooves and posts were needed. Regular ridge grooves with standard 1:1 aspect ratios were designed along with 1μm diameter posts because they are the smallest feature size that can still retain high resolution during the lithography process. Because ridge groove features yielded positive results in experiments while posts are hypothesized to yield positive results for photoreceptor marker, a new design combing both features was created. Designs for these masks were drawn up in the L-Edit CAD program and sent to Toppan Photomask, Inc. for manufacturing of the photomask. This particular mask, is a 5" x 5" x 0.090" plate with 0.25μm spec and ±0.075μm tolerance. Defect inspection is at 2μm at 2DPSI with chrome on the "ridge" feature and clear transparency on the "chrome" feature.
Figure 4-2: L-Edit CAD drawing of new designs on mask. (A) 0.50um 1:1 ridge-grooves (B) 1.00um 1:1 ridge-grooves (C) 0.75um 1:1 ridge-grooves (D) 1.00um diameter posts draw as squares in L-Edit due to grid-specs

Figure 4-3: Optical image of chrome mask by Toppan

4.2.2 Microfabrication

The exact same steps are done as in the previous fabrication process for the posts-only and ridge groove-only features. For the feature combining both ridge grooves and posts, silicon
oxide-coated wafers with ridge grooves already patterned were spin-coated with a layer of thicker photoresist (S1822, Shipley) at 3500 rpm for 20s to cover the groove depth as well as to leave a layer of photoresist on top of the ridges. The ridge-groove embedded wafer with this 5000Å of photoresist was then exposed with the post portion of the chrome mask. Once standard development and etching were done, the desired ridge groove structure with posts in between was produced.

Film fabrication procedures were identical to previous procedure with 661W cells. (Figures of results will be shown under results section)

4.2.3 Cell Staining

PCL thin films were gathered 7 days after seeding and fixed in 4% paraformaldehyde (Sigma) for 20 mins before going through the immunocytochemistry process. Samples were rinsed 3 times at 5 minutes intervals in PBS and blocked with 10% goat serum (Invitrogen), 3% BSA (Sigma) and 0.1% Triton-X (Sigma) for 60 minutes. Samples were then incubated overnight after staining of primary antibodies with Nestin (BD, 1:200), Sox2 (Chemicon, Temecula, CA, USA, 1:200), Ki67 (Chemicon, 1:100), Pax6 (Abcam, Cambridge, MA, USA, 1:100), crx (Santa cruz, Santa Cruz, CA, USA, 1:100), Recoverin (Chemicon, 1:1000), Rhodopsin (Chemicon, 1:100), S-opsin (Abcam, 1:100), Glial fibrillary acidic protein (GFAP, Chemicon, 1:400), Green fluorescent protein (GFP, Abcam, 1:500), and GFP (Chemicon, 1:500). In creating a negative control for staining, normal serum was used. Secondary antibody conjugates of Cy2 (1:100) or Cy3 (1:400) were used afterwards for 60 minutes at room temperature. Nuclei of cells were counter-stained with anti-fade DAPI reagent before being looked at under fluorescent microscopes. Note: All staining was performed at the same time to eliminate experimental bias.

Optical density percentage (%OD) of various photoreceptor markers such as cone-rod homeobox (CRX), rhodopsin and recoverin were tested on cells seeded on non-patterned glass
(used as control), non-patterned PCL, and patterned PCL. The optical density measurement reading will be explained in the result section.

CRX is a gene in humans that encodes proteins that are photoreceptor-specific transcription factors which are vital for photoreceptor cells to differentiate. This protein encoded by the CRX gene helps maintain normal cone and rod function in the retina.

Rhodopsin is a pigment consisting of opsin protein in the retina that signals photoreceptor formation and allows the eyes to see in low light, while Recoverin is a protein in photoreceptor that regulates eye recovery after exposure to light. Any upregulation of the above three photoreceptor markers would represent the differentiation of these RPCs into photoreceptor cells.

4.2.4 SEM Preparations

The same steps were taken for SEM preparation for previous 661W cells. While half the samples were used for staining images, the other half were fixed for SEM images. Primary fixative of 3% Glutaraldehyde in Sucrose-Cacodylate buffer (1.5mL of 50% Glutaraldehyde in 25mL) was prepared along with Sucrose-Cacodylate buffer containing 0.1M sodium cacodylate and 0.1M sucrose (2.14g sodium cacodylate and 3.424g sucrose in 1L sucrose-cacodylate buffer).

Fixed cell samples were placed with sucrose-cacodylated buffer and left for 5 minutes. Buffer was then removed and the rinse procedure was repeated for an additional 5 minutes. Cell samples were then dehydrated by adding and replacing solution of ethanol in a graded series of concentrations of 35% for 10 minutes, 50% for 10 mins, 70% for 10mins, 95% for 10 mins and 100% for 10 mins.

4.2.5 Quantification of RPC Morphology
RPCs were analyzed after they were seeded and cultured for 7 days, after which their morphology as well as their marker expressions were compared against each other. For samples prepared for scanning electron microscopy, images were taken at pre-determined positions. These images were then analyzed using ImageJ for their cell body elongations, alignments to a single direction, cell surface area in opposite of scaffolds, and measurements of dendrites. As with 661W cells, the outline of each individual cell was traced and a best-fit ellipse was automatically fitted to that outline. For cell surface area, the wand tool in ImageJ was utilized to capture the cell surface area in between cell edges who has a different intensity that the film surface.

Figure 4-4: (A) Outline of cell body area measured in imageJ (B) Outline of best-fit ellipse and its orientation angle measured in imageJ

4.2.6 Statistical Analysis

The same statistical analysis mentioned in the previous chapter was used in this case.

4.3 Results

4.3.1 Manufactured Chrome Mask from Toppan
Chrome/transparent features on photomask were checked under a bright field microscope (Optical Nikon Optiphot 200) for size accuracy and defects. Measurements were done with the ImagePro program to verify how closely features on mask match those in design:

<table>
<thead>
<tr>
<th>Chrome Masks (um)</th>
<th>1um diameter posts</th>
<th>0.50um Ridge Grooves</th>
<th>0.75um Ridge Grooves</th>
<th>1.0 um Ridge Grooves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.21 ± .11</td>
<td>0.45 ± 0.01</td>
<td>0.714 ± 0.01</td>
<td>0.98 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 4-5: Images of chrome mask features measured by optical microscope and characterization of these features

4.3.2 Result of Microfabrication

Micropatterned silicon wafers were fabricated to contain all five features of 1um diameter posts and 0.50um wide ridge grooves, 0.75um wide ridge grooves, 1.0um wide ridge grooves, and combined ridge groove and posts using photolithography techniques and deep reactive ion etching. All features are 1um in height as well. After the etching and piranha clean, the designed 1.0um posts, 0.50um ridge grooves, 0.75um ridge grooves, and 1.0um ridge grooves had (in µm) average dimensions of 1.00 ± 0.11, .49 ± .04, 0.75 ± 0.14, and 1.10 ± .20, respectively.

<table>
<thead>
<tr>
<th>Silicon Wafers (um)</th>
<th>1um diameter posts</th>
<th>0.50um Ridge Grooves</th>
<th>0.75um Ridge Grooves</th>
<th>1.0um Ridge Grooves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.00 ± 0.11</td>
<td>.49 ± 0.04</td>
<td>0.75 ± 0.14</td>
<td>1.10 ± .20</td>
</tr>
</tbody>
</table>

Figure 4-6: Characterization of physical features on silicon master mold
Figure 4-7: Images of silicon wafers developed from photolithography

Figure 4-8: SEM images of (A) nano-posts (B) 0.75um ridge-grooves (C) 0.50um ridge-grooves and (D) 1.00um ridge-grooves in silicon master mold
Figure 4-9: SEM image of combined ridge-grooves and posts on silicon master mold. Ridges and grooves, and pits are labeled. Films peeling off of this mold will have ridges, grooves, and posts.

4.3.3 Thin Film Fabrication

Each completed silicon wafer master mold was placed in 2% soap solution for 300s for the sacrificial layer to develop followed by spin-casting of dissolved PCL to the silicon wafer master mold, resulting in a thin layer of polymer coating on the silicon wafer surface. Following baking at 70°C for 15mins, submerging into deionized water allowed the PCL layer to be peeled from the surface. PCL films were fabricated both from patterned wafers as well as smooth blank wafers. Films peeled from smooth blank wafers were used as controls to compare the effects of physical cues. Film thickness depends on spin speed; in this case, 1500 rpm was used to produce a 5μm thickness. As for quality of topographical cues, the designed 1.0μm posts, 0.50μm ridge grooves, 0.75μm ridge grooves, and 1.0μm ridge grooves had (in μm) average dimensions of 1.087 ± 0.06, 0.48±0.065, 0.75±0.105, and 1.14 ± .05, respectively.
Figure 4-10: Image of thin film spun off of silicon master mold

Figure 4-11: SEM images of (A) nano-posts (B) 1.00um ridge-grooves (C) 0.75um ridge-grooves and (D) 0.50um ridge-grooves on silicon master mold
4.3.4 Cell Morphology

4.3.4.1 Cell Elongation

The mRPCs were cultured on both blank and patterned thin film surfaces to investigate the influence of substrate topography on morphology and various physical attributes. Cells on both patterned and smooth blank films were looked at under SEM and analyzed with ImageJ software. For cell morphology, cells on smooth blank films maintained similar circular cell body with no biased elongation in any one direction, with an average elongation of 0.48. For cells on post film, the cell body becomes more circular than those on blank films, with average elongation of 0.28. For cells on ridge groove films, the cell body elongates considerably in a particular direction (usually more parallel to the ridge groove), with an average of 0.57. With combined features of ridge grooves with posts in between ridges, the elongation was 0.47.

Overall, it is most probable that these retinal progenitors' morphologies are based on contact
guidance from their microenvironment, and that the cells stretch in the direction that has the surface area they can “feel”. This would explain the change in elongation in the direction of ridge grooves as well as the increased circularity on circular posts. For the combined features, cells most likely felt the ridge grooves going in one direction and the posts going in a perpendicular direction, which led to cell bodies stretching two directions perpendicular to each other. This ultimately slows down the elongation in one particular direction.

![Box and whisker plot of RPC elongation among cells on blank control film, posts, ridge-grooves, and combined ridge grooves and posts](image)

Figure 4-15: Box and whisker plot of RPC elongation among cells on blank control film, posts, ridge-grooves, and combined ridge grooves and posts

![Graph showing RPCs Average Elongation](image)

Figure 4-16: Average RPC elongation and standard deviation among cells on blank control film, posts, ridge-grooves, and combined ridge grooves and posts
4.3.4.2 Cell Alignment

While the micro-patterning of the substrate produced a significant effect on cell elongation, it also has a strong effect on alignment of differentiating cells. Quantitative analysis of cell alignment was performed by measuring the angle between the cell's major axis against the direction of the ridge grooves for ridge grooves patterned film, and for a horizontal x-axis direction for posts and control films as described in the previous materials and methods section. Cells cultured on smooth blank films and posts films had average alignment to film's x-axis at 50.4° and 52.24° respectively while those on ridge groove films and combined features films showed great alignment to the direction of the ridge grooves with average alignment angles of 9.5° and 13.4°, respectively. The cells seeded on ridge-grooves patterns were significantly closer to perfect alignment than those on smooth films. The bar chart below also demonstrated most cells on control blank films and post films did not have inclination towards any angle since the distribution of cells across most angle alignment was low (low standard deviation among blank and post films).

As with elongation in the previous section, contact guidance with ridge groove probably led cells to align towards a particular direction. Posts had same random alignments as blank films since posts were equal distant from each other in 6 different directions instead of concentrating all in one direction. For the combined features, cells probably followed contact
guidance from ridge grooves but their degrees of alignment was also reduced by the contact guidance of posts going in a perpendicular direction.

Figure 4-18: Box and whisker plot of RPC alignment angles among cells on blank control film, posts, ridge-grooves, and combined ridge grooves and posts

<table>
<thead>
<tr>
<th>α = 0.05</th>
<th>Control Vs Post</th>
<th>Control Vs Ridge-Grooves</th>
<th>Control Vs Combined</th>
<th>Posts Vs Ridge-Grooves</th>
<th>Posts Vs Combined</th>
<th>Ridge Grooves Vs Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Value</td>
<td>P = 0.5005</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P = 0.0323</td>
</tr>
<tr>
<td>Significance</td>
<td>not statistically significant</td>
<td>extremely statistically significant</td>
<td>extremely statistically significant</td>
<td>extremely statistically significant</td>
<td>extremely statistically significant</td>
<td>statistically significant</td>
</tr>
</tbody>
</table>

Figure 4-19: Statistical testing was done at α = 0.05 to see whether the P value was small enough for the cell alignment average differences to be deemed statistically significant.
Figure 4-20: Quantification of alignment angles of RPCs. RPCs skewed more towards perfect alignment when on ridge-grooves film than on control film. Cells on control films also had even distribution of cells orienting towards all different directions (sample space was near ~400).

Figure 4-21: Standard Deviation of cells aligning towards any 10° range. Because the standard deviation for cells and control posts are relatively low, there is no single 10° range in which cells prefer to align to.
4.3.4.3 Cell Area and Perimeter

As for cell area and cell perimeters, cells on control, posts, and ridge-grooves were analyzed. (Cells on combined ridge-grooves and posts were not analyzed at the time of this thesis.) Cell were cultured on the micropatterned substrate for 7 days showed decreased cell surface area (average of 85.3μm² for ridge groove films and 89.2μm² for posts films) than cells on smooth films (average of 131.3μm²). There were no significant differences in cell area among cells seeded on patterned films. The same result also applies to the cell outline perimeters as well with control, posts and ridge groove films having average perimeters of 130μm, 350μm, and 320μm, respectively.

![Box and whisker plot of RPCs body area when seeded on control, posts, and ridge-grooves film.](image)

Figure 4-22: Box and whisker plot of RPCs body area when seeded on control, posts, and ridge-grooves film.
Figure 4-23: Averages of RPC body area among cells on ridge-grooves (156um²), posts (121um²) and smooth films (110um²)

<table>
<thead>
<tr>
<th></th>
<th>Control Vs. Ridge-Grooves</th>
<th>Control Vs. Posts</th>
<th>Ridge-Grooves Vs. Posts</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Value</td>
<td>P = 0.0087</td>
<td>P = 0.0329</td>
<td>P = 0.3903</td>
</tr>
<tr>
<td>Significance</td>
<td>Extremely statistically significant</td>
<td>statistically significant</td>
<td>Not statistically significant</td>
</tr>
</tbody>
</table>

Figure 4-24: Statistical testing was done at α = 0.05 to see whether the P value was small enough for the cell area average differences to be deemed statistically significant
Figure 4-25: Box and whisker plot of RPC perimeters on various patterned films. As stated before, red line is median blue box marks 1st and 3rd quartile.

![Box and whisker plot of RPC perimeters on various patterned films](image)

Figure 4-26: Averages of RPC body perimeters among cells on control (1178um), posts (1201um) and ridge-grooves films (1215um)

![Bar chart showing averages of RPC body perimeters](image)

<table>
<thead>
<tr>
<th></th>
<th>Control Vs. Ridge-Grooves</th>
<th>Control Vs. Posts</th>
<th>Posts Vs Ridge-Grooves</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Value</td>
<td>P = 0.9143</td>
<td>P = 0.9361</td>
<td>P = 0.9514</td>
</tr>
<tr>
<td>Significance</td>
<td>Not statistically significant</td>
<td>Not statistically significant</td>
<td>Not statistically significant</td>
</tr>
</tbody>
</table>
4.3.4.4 Cell Processes Extension

Sub-micron topographical cues' effects on cell processes were also analyzed. Cell processes (axons and dendrites) were measured for their length and angles. Length is defined as the length from the cell body to the end of the process, not the perimeter. Number of processes was not factored in since the measurement is the average of all processes. Specifically, the end angle, angle between processes directions and ridge groove direction or angle between processes directions and x-axis of thin film were measured in all three sets of films and compared for any significant difference.

For processes lengths, there does not seem to be significant difference among cells on blank control films, posts film or ridge groove films, which had average processes length of
35.8\mu m, 35.6\mu m and 28.6\mu m, respectively. For processes' end angles, there was no significant difference between control and posts (both around 29^\circ C), but there were substantial difference between non-ridge grooves and ridge grooves (at 9.6^\circ C). This signified that ridge groove topography might cause directional changes on cell processes in addition to just cell body alignment.

![Box and whisker plot of RPC processes](image)

Figure 4-29: Box and whisker plot of RPC processes
Figure 4-30: Averages of RPC processes lengths among cells on control, posts, and ridge-grooves films. Standard deviations are also included.

Figure 4-31: Box and whisker plot of RPC processes' alignment angles at the ends
4.3.4.5 Cell Immunocytochemistry

The mRPCs were cultured on oxygen-plasma treated PCL substrates surfaces to investigate the influence of sub-micron topography on cell growth and differentiation. Approximately 15 samples of each type were examined. In general, cells seeded on patterned PCL thin films showed significantly greater immunoreactivity for all three markers than cells seeded on glass. This is shown through a measurement of optical density. Optical density measures the transmittance of the optical medium given a specific wavelength. It is at a higher percentage when the stain has identified a large number of photoreceptor markers. For cone-rod homeobox, cells on glass substrates had around a 2% optical density, while cells on smooth PCL had slightly higher optical density at 18%. Cells seeded on ridge-groove PCL and post PCL had significantly higher optical density at 51% and 46%, respectively. For Recoverin, cells on glass substrate had around a 9% optical density. Cells on smooth and ridge-groove PCL had slightly higher optical densities at 32% and 33% respectively, while post PCL had significantly higher optical density at 51%. For Rhodopsin, cells on glass substrate had around a 3% optical
density. Cells on smooth and post PCL had slightly higher optical density at 17% and 23% respectively, while ridge-groove PCL had significantly higher optical density at 53%.

Figure 4-33: Stained images for CRX, Recoverin, and Rhodopsin on smooth and patterned thin films. Scale bar = 100um
These above results demonstrate the importance of biomaterial in instigating mRPC differentiations. All categories in the chart with ‘*’ denotes statistically significance in differences. Thus, a total of 6 comparisons had significance difference in the outcome while the non-statistically significance one will need more studies. From all this, it can also be deduced that different micro topographical cues induce different photoreceptor marker upregulation. In this case, both topographical cues instigated a rise in CRX gene while posts shape increased Recoverin and ridge groove shape increased Rhodopsin. Stem cell markers were stained in this experiment, but their optical density percentage showed no conclusive results.

4.4 Discussions

The means to organize and differentiate mRPCs in a controlled manner enhance mRPC organization after transplantation to the retina is extremely vital to the development of tissue engineering and regenerative medicine, especially pertaining to the eye. This current thesis project as well as the current and future work in this project will hopefully contribute more to the research surrounding this area.
The results from this project revealed the effects that sub-micron topographical cues have on mRPCs morphology and cell differentiation. This thesis has confirmed that sub-micron structures can be microfabricated in biodegradable PCL thin films with high accuracy and reliability and that microfabricated PCL thin films can induce organization of mRPCs in vitro to some degree. While this experiment only looked at cells cultured for 7 days or before, mRPCs culture for beyond 7 days might exhibit even more organization than shown here in this chapter. For differentiation markers, physical cues were able to promote morphological differentiations of mRPCs and upregulate expression of photoreceptor markers. Both the biomaterial and physical shapes of cues were factors in contributing to the higher expressions levels. While not every photoreceptor markers were studied, the most common ones (CRX, Rhodopsin, and Recoverin) were with positive results. Future studies of this experiment might include looking into more photoreceptor markers as well as studying more variations of physical shapes.

4.4.1 Microfabrication and Film Development Reliability

This project expands on previous work that researched various ways of developing micro-architecture on tissue culture plastic and tested for cell responses in a wide range of geometries. It is from following these studies and combining photolithography techniques with film development techniques that we have been able to fabricate specific sub-micron patterns for instigating favorable responses from mRPCs. Although the number of geometric patterns tested was limited, features as small as 500nm were successfully transferred from designed patterned masks onto silicon oxide wafers and then transferred onto thin PCL films without the loss of a substantial amount of resolution. Because this thesis project also expands on past studies that were done on the biocompatibility of PCL and the techniques that can best develop the most favorable film, we were able to develop a thin film substrate that can hold these patterns while remaining less than 5μm thick such that together with the cells, they will be able to fit inside the retinal layer and degrade within 2 years in the body. Although the films were
never tested in vivo for this project, the effects of micro-patterned physical cues on mRPCs for alignment, elongation, area, and perimeters were quantified. Thin film sub-micron topography produced significant effects on cell morphology in that it was able to align cells in a specified range of a certain direction, cause cell elongation, and decrease area/perimeter of cells. Furthermore, the effects of micro-patterns were able to restrict the angle at which processes grow from a cell body. This change in cell morphology, especially increase in elongation might perhaps be due to physical transformation from stem cell to photoreceptor cell since all photoreceptor cells tend to gravitate towards having higher elongation. If this is true, cell manipulation techniques may perhaps be used as a process in which mRPCs can be molded into the shape of photoreceptors. The cell morphology responses are also a strong indication that in the absence of any biochemical signaling, mRPCs behave according to their physical surroundings.

4.4.2 Cell Staining

The analysis done by Schepens on cell differentiation demonstrates the effects and biocompatibility PCL has over other material (glass in this case) during a 7 day period. These mRPCs were isolated from retinas of postnatal day 1 eGFP transgenic C57BL/6 mice and kept as neurospheres in culture medium before being seeded onto thin films. While stem cell differentiation potential in past studies have yielded mixed results, a 7-day culture of mRPCs showed these cells to be differentiating more rapidly on PCL thin films than on glass thin films and more rapidly on micro-patterned films than on smooth films. While the exact theory behind this phenomenon is still being explored, it is very possible that the higher levels of %Optical Density in these three photoreceptor markers mentioned above are caused by the difference in film material stiffness in addition to physical shapes. Such is the case for other types of stem cells. For example, researchers have found that bone marrow stem cells begin to differentiate along different paths based only on the environment stiffness while being denied of any
chemical signaling. Other possible physical features that cause specific differentiation may include tension or compression along the cell body. As with fibroblasts mentioned in Chapter 1, cells may differentiate down a specific path once cell body reaches a certain level of uniaxial stress. These areas have yet to be fully researched and are beyond the scope of this particular thesis project. However, the next step in this immunocytochemistry process currently underway involves staining for the same photoreceptor markers in thin films consisting of a combination of both ridge grooves and posts to gauge if an even higher %Optical Density is seen for all three markers.
Chapter 5: Conclusion

5.1 Current Progress

Improving treatment options for retinal transplantations can help thousands of people who suffer from these retinal diseases and who are seeking cures from the field of regenerative medicine. The goal of this thesis project is to take a small step towards seeing how microfabrication and scaffolding can help in delivering cells to areas affected by retinal degeneration by testing for favorable morphology changes in retinal stem cell structures and for increase of differentiation potential when culturing cells on these scaffolds. While the overall goal of this project is still years away from completion, many current ongoing experiments are making good progress in getting closer this goal. Current projects include applying the same process mentioned in this thesis but on human retinal progenitor cells (harps) as well as iPSc. Seeing results from these two sets of cells will give us better insights into which physical micro features work best and whether the results we got above only apply to mouse retinal cells or also apply universally to all retinal stem cells and induced pluripotent stem cells. Additionally, data from these experiments can also be used in developing future devices for retinal stem cell culture.

5.2 Future Work

The next steps after these experiments would be to use the data and lessons learned from these experiments to develop 3D scaffold. Once this 3D scaffold is tested to work well in instigating cell differentiations into photoreceptors, it will be tested to insure degradation periods as well as mechanical stiffness/structural integrity before finally being committed to in vivo experiments. This scaffold would be implanted into a mouse's retina for an extended amount of time to test for the retina's reactions to the scaffold and whether cells behave the same as when
they are cultured *in vitro*. In addition, more research will probably be done to seek better ways of developing more complex nanostructures on implantable scaffolds for other areas of the body.
Works Cited


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