Effect of Extracellular Matrix (ECM) Protein Micropatterns on the Behavior of Human Neuroblastoma Cells

Ishwari Poudel
University of Nebraska-Lincoln, paudelishwori@hotmail.com

Follow this and additional works at: http://digitalcommons.unl.edu/engmechdiss

Part of the Bioelectrical and Neuroengineering Commons, Biological Engineering Commons, Biomaterials Commons, Biomechanical Engineering Commons, Biomedical Devices and Instrumentation Commons, Molecular, Cellular, and Tissue Engineering Commons, Nanoscience and Nanotechnology Commons, and the Polymer and Organic Materials Commons

http://digitalcommons.unl.edu/engmechdiss/24
Effect of Extracellular Matrix (ECM) Protein Micropatterns on the Behavior of Human Neuroblastoma Cells

By

Ishwari Poudel

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Engineering Mechanics

Under the Supervision of Professor Jung Yul Lim

Lincoln, Nebraska

November, 2011
Effect of Extracellular Matrix (ECM) Protein Micropatterns on the Behavior of Human Neuroblastoma Cells

Ishwari Poudel, M.S.

University of Nebraska, 2011

Adviser: Jung Yul Lim

Recent advances in patterning techniques and emerging surface microtechnologies have allowed cell micropatterning to control spatial location of the cells on a surface as well as cell shape, attachment area, and number of contacting neighbor cells. These parameters play important roles in cell cellular behaviors. Cell micropatterning has thus become one of the most important strategies for biomedical applications, such as, tissue engineering, diagnostic immunoassays, lab-on-chip devices, bio-sensing, etc., and cell biology studies as well. For neuronal cells, there have been attempts to distribute neuronal cells on specific patterns to control cell-to-cell interaction. However, there have been very limited understanding on the effects of micropattern size and specific ECM proteins used for patterning on neuronal cell behavior. In this work, in-vitro neuronal cell patterning was performed using various ECM protein lane widths with or without neuronal biochemical factor to investigate neuronal cell response to controlled microenvironments.

We have developed a technique to organize cells into pre-assigned boundaries while maintaining their original properties of growth, proliferation, and differentiation.
Soft lithography and microcontact printing were used to pattern arrays of Collagen-I ECM lanes with 5, 10, 20, 30, and 40 µm width separated by 50 µm gap. PLL-g-PEG was flooded before culturing SH-SY5Y cells. Cells did not prefer small adhesive lanes, but attached on as small as 5µm ECM lanes if cell-repellent backfilling was utilized. On 5 and 10 µm lanes, cell and nuclear growth was constrained as compared to unpatterned control, and wider lanes. Cells showed near perfect orientation along the adhesive lanes for 5 and 10 µm width lanes. With increasing lane width, neuronal cell orientation was influenced adversely, e.g., increased deviation from the patterning direction. When stimulated by retinoic acid (RA), cells patterned on 5 and 10 µm lanes showed well-developed, long neurites along the protein pattern connecting neighboring cells. The neurite length was shorter on wider lanes. Our data may provide a new insight for neuronal tissue engineering on how to regenerate damaged neurons using more controlled physical-biochemical environments.
Acknowledgements

I would like to acknowledge my advisor Dr. Jung Yul Lim for his encouragement, endless support and guidance throughout the years. These studies would not have been possible without his outmost patience and trust while developing and establishing a fully functional live cells patterning protocol from the scratch. He provided me with ample freedom on exploring alternative studies, good working ethics as well as working environment in the lab, which helped me to stay focused and dedicated throughout the studies. Dr. Lim’s highly professional advises and in-depth knowledge in the subject matter were of great assistance for the completion of these studies.

I would like to thank Dr. Jung Yul Lim, Dr. Li Tan, and Dr. Linxia Gu for serving on my thesis committee and for their assistance and constructive comments.

I would like to thank Dr. Jeong Soon Lee and all of my lab members for their technical support.

I am heartily thankful to Dr. Li Tan for letting me use his lab and photolithography facility endlessly. I would also like to thank Dr. Raffet Velarde for providing me technical assistance and help in using Biomechanics, Biomaterials and Biomedicine (BM³) instrumentation facility.

Last but not least, I would like to thank my parents and family members for their continuous moral support and blessings throughout my stay here at the US.
# Table of Contents

Chapter 1: Introduction .......................................................................................................................... 1
  Literature review .......................................................................................................................... 1
    Effect of ECM on cellular Behavior ........................................................................................ 2
    Alteration of cell behavior through size, shape, and interconnectivity .................................... 3
    Techniques for chemical patterning, cell patterning, and cellular isolation ............................. 7
    Cellular confinement in designated geometry ......................................................................... 8
  Research Objectives ................................................................................................................... 12
  Organization of Thesis ............................................................................................................... 12

Chapter 2: Photolithography ................................................................................................................. 14
  Soft lithography ......................................................................................................................... 15
    Materials for soft lithography ................................................................................................. 15
    Benefits of using an elastomeric stamp ................................................................................. 17
    Failure of an elastomeric stamp ............................................................................................. 18
    Microcontact Printing ......................................................................................................... 20
  Experimental section .................................................................................................................. 21
    Materials and chemicals ......................................................................................................... 21
    Mask Design .......................................................................................................................... 22
    Master fabrication .................................................................................................................. 22
    Stamps Fabrication............................................................................................................. 24
  Master and Stamps Characterization .......................................................................................... 25
  Substrate preparation ................................................................................................................. 26
    Surface modification of glass slides/coverslips and PDMS membrane ................................. 26
  Production of micropatterned surfaces ...................................................................................... 27
    Chemicals and proteins ......................................................................................................... 27
    Stamps and biosubstrate surface modification ......................................................................... 27
    Microcontact printing........................................................................................................... 28
    Passivation of non-printed areas through PLL-g-PEG backfilling ........................................ 28
    UV Sterilization of micropatterned substrates ....................................................................... 29
  Cell culture ................................................................................................................................ 29
  Microscopy .................................................................................................................................. 29
  Results ........................................................................................................................................ 30
List of Figures

Figure 1-1: Cell Shape Drives hMSC Commitment (A) Brightfield images of hMSCs plated onto small (1024 µm²) or culture on 1024 or 10000 µm² islands after 1 week in growth or mixed media. Lipids stain red; alkaline phosphatase stains blue. (B) Percentage differentiation of hMSCs plated onto 1024, 2025, 1000 µm² island after 1 week of culture in mixed media. Scale bar = 50 µm. (McBeath et al., 2004).

Figure 1-2: Amount of biofilm adhering to stamped surfaces of PDL and flooded regions of PEG in culture over the duration of one month. Shown at each data point is the amount of biofilm (µg/cm²) adhered to PDL or PEG modified substrates. For PDL substrates, there is a dramatic increase in biofilm adhesion between 2 and 3 weeks, whereas only a nominal increase is observed on PEG modified substrates (Branch et al., 2000).

Figure 2-1: Polymerization of a mixture of PDMS prepolymer and curing upon application of heat (Eeva Lääniläinen, 2006).

Figure 2-2: Barrier tolerance of an elastomeric stamp and a hard stamp.


Figure 2-4: Diagram illustrating different failure events of an elastomeric stamp (Younan Xia and George M. Whitesides, 1998).

Figure 2-5: diagrammatic illustration of photolithography, soft lithography and microcontact printing.

Figure 4-1: Schematic diagram of nuclear orientation measurement. Straight line parallel to protein pattern was regarded as zero degree, and the right/left deviation from the reference line was measured as the orientation of that particular nucleus.

Figure 4-2: measurement of cellular area using imageJ image analyzing software. (A) Original RGB image obtained with fluorescence microscope, (B) after enhanced contrast among the colors, (C) after application of threshold, (D) after adjustment of threshold colors, (E) after selection of cellular area, and (F) selection of only cell area excluding nuclear areas.

Figure 4-3: particle analysis fluorescence images to calculate nuclear area using imagemJ image analyzing software. (A) Original image of nuclei obtained with fluorescence microscope of DAPI staining, (B) after enhanced contrast among colors, (C) after application of threshold, (D) after adjustment of threshold colors, (E) after selection of cellular area, and (F) outline with numbered areas obtained after particle analysis of nuclei.

Figure 4-4: Neurite extension measurement using NeuronJ add-on of ImageJ image analyzing software.

Figure 4-5: Fluorescence images of ECM protein patterns and PLL-g-PEG backfilling on tissue culture plate surface. FITC conjugated collagen-I lanes (A) 5 µm (B) 20 µm and (C) 5 µm FITC
conjugated collagen-I lanes backfilled with TXRD conjugated PLL-g-PEG (Scale bar = 50 µm).

Figure 4-6: SH-SY5Y cells on collagen-I ECM patterns on day 1 without any backfilling. (A) 5 µm, (B) 10 µm, ................................................................. 48

Figure 4-7: SH-SY5Y cells on collagen-I ECM patterns on day 1 with PLL-g-PEG backfilling. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D) 30 µm, and (E) 40 µm proteins lanes separated by 50 µm gap (scale bar = 50 µm). ................................................................. 49

Fig. 4-8: SH-SY5Y cells on collagen-I ECM pattern day 7 without any backfilling and no RA. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D)30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm).................................................................................. 50

Figure 4-9: SH-SY5Y cells on collagen-I ECM pattern on day 7 without any backfilling and with 10 µM RA (A) 5µm, (B) 10 µm, (C) 20 µm, (D)30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm).................................................................................. 51

Figure 4-10: SH-SY5Y cells on collagen-I ECM pattern on day 7 with backfilling but without RA. (A) 5 µm, (B) 10 µm, (C) 20µm, (D)30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm). ................................................................................................................ 52

Figure 4-11: Optical images of SH-SY5Y cells on collagen-I ECM pattern on day 7 with backfilling and with 10 µM RA. (A) 5 µm, (B) 10 µm, (C) 20µm, (D)30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm)................................................................................................................. 53

Figure 4-12: Fluorescence images of SH-SY5Y cells on collagen-I ECM pattern day 7 without RA. (A) 5 µm, (B) 10 µm, (C) 20µm, (D)30 µm, (E) 40 µm, and (F) control. Red = actin cytoskeleton, blue = nucleus (scale bar = 50 µm)................................................................................................................. 53

Figure 4-13: Fluorescence images of SH-SY5Y cells on collagen-I ECM pattern on day 7 with 10 µM RA. (A) 5 µm, (B) 10 µm, (C) 20µm, (D)30 µm, (E) 40 µm, (F) control. Red=actin cytoskeleton, blue= nucleus (scale bar = 50 µm)................................................................................................................. 54

Figure 4-14: Analysis of cellular and nuclear spreading area for SH-SY5Y neuroblastoma cells on day 7 without supplement of retinoic acid. ................................................................................................................ 55

Figure 4-15: Analysis of cellular and nuclear spreading area for SH-SY5Y neuroblastoma cells at day 7 with supplement of retinoic acid. ................................................................................................................ 56

Figure 4-16: Analysis of nuclear orientation for SH-SY5Y neuroblastoma cells on day 7 without retinoic acid. ................................................................................................................ 57

Figure 4-17: Analysis of nuclear orientation for SH-SY5Y neuroblastoma cells on day 7 with 10 µg/mL retinoic acid. ................................................................................................................ 58

Figure 4-18: Neurite extension as a function of ECM lane width. ................................................................................................................ 59

List of Tables

Table 1: optimized parameters for microcontact printing............................................................... 34
Chapter 1 : Introduction

Transformation of synthetic chemical features on biosubstrates has provided methods to engineer cell-biomaterial interfaces to control their interactions. In our body, cells respond to chemical and mechanical cues through extracellular matrix protein, or mechanical and chemical features at the cell-biomaterial interfaces. Understanding of such a complex mechanism requires a systematic study. Chemical surface patterning, topography and chemical patterns created on the surfaces can provide user-defined and well characterized substrates as a logical approach to investigate cellular responses to surfaces and microenvironments around the cells. Cell micropatterning technique, a type of chemical surface patterning, has been used extensively to understand the responses of cells on biomaterial surfaces, chemical and mechanical cues.

Literature review

Cell micropatterning is a method to control living cells organization on various substrate surfaces. Current methods of cell micropatterning fall into three categories based on the strategy used to seed cells: (i) on a chemically patterned surface, (ii) on a topographically patterned surface, or (iii) directed delivery of cells onto discrete regions of a substrate (Joseph M. Corey and Eva L. Feldman, 2003). One of the most important aspects of cellular micropatterning is to understand the basic mechanisms and features of cells and tissues. In the early stage, cellular micropatterning was mainly focused on patterning neuronal cells to mimic the in-vivo architecture in-vitro (Joseph M. Corey and Eva L. Feldman, 2003), but recent advances in patterning techniques and emerging surface nano/microtechnologies have allowed cell micropatterning to control spatial location of the cells on a surface as well as the shape, attachment area, and number of contacting cells for each individual cell (Joseph M. Corey and Eva L. Feldman, 2003; Wang & Ingber, 1994). These parameters have been found to be quite important for cell survival (Wang & Ingber, 1994), growth (Wang & Ingber, 1994), proliferation (Chen,
Mrksich, Huang, Whitesides, & Ingber, 1997; Discher, Mooney, & Zandstra, 2009), cytokinesis (Chen et al., 1997), differentiation (McBeath, Piro, Nelson, Bhadriraju, & Chen, 2004; Yang, Co, & Ho, 2005a), cell polarity (Chen et al., 1997; Frimat et al., 2010), and cell migration (Chen et al., 1997; Rosenthal, Macdonald, & Voldman, 2007) on a single cell level. For this reason, cell micropatterning has become one of the most important methods for cell biology research (Chen, Mrksich, Huang, Whitesides, & Ingber, 1998; Joseph M. Corey and Eva L. Feldman, 2003; McBeath et al., 2004) and biotechnology applications, such as, tissue engineering, diagnostic immunoassays, lab-on-chip devices, cell metabolism studies and bio-sensing (Lauer, Klein, & Offenhauser, 2001; Wang & Ingber, 1994). However, selection of appropriate biomolecules and their integration into proper micropatterns are very important for long term viability of cells on the surfaces. Therefore, designing a robust control of physical, chemical, electrical and mechanical microenvironments is necessary for safe and effective regeneration of functional tissues (Discher et al., 2009; Wang & Ingber, 1994) as the surrounding microenvironment has direct effect on cells and their behavior (Sgarbi et al., 2004).

**Effect of ECM on cellular Behavior**

Cells conform to the pattern that they are confined to and as a result develop unique intracellular architecture which in turn regulates differentiation, proliferation, growth, and cell survival. Basement membranes act as a platform to define cellular structures throughout human body. Extracellular matrix protein components, for example, fibrous collagen, proteoglycans, laminin, fibronectin and hyaluronic acids of basement membranes, may play an important role to overlay cellular structures in our body (Flemming, Murphy, Abrams, Goodman, & Nealey, 1999). Exploiting this information, various synthetic ECM proteins have been used to investigate the effect of surface chemistry and topography on cell and tissue functions. Coopman et al. (Coopman et al., 1991) studied the effect of laminin and hyaluronic acid on cell behavior and reported that laminin prevents cell migration while hyaluronic acid promotes cell migration but inhibits cell-cell adhesion (Coopman et al., 1991). Adhesion of cells with excessive ECM
proteins causes in cytoskeletal rearrangement, which promotes replication of cells (Carnegie & Cabaca, 1993). Studies conducted in various surfaces using ECM proteins have reported that attachment of cells is higher and faster in coated surfaces as well as the area also found to be significantly larger (Buzanska et al., 2009; Corey et al., 2010). These cellular behaviors are regulated through basement membrane by activating plasma membrane integrin receptors, such as, RGD that binds to ligand on the basement membrane (Chen et al., 1997; Clark & Brugge, 1995; Hynes, 1992; Juliano & Haskill, 1993; Ruoslahti, 1997).

Similarly, integrin receptors have been reported as signaling molecules for various pathways to control cell growth and survival (Chen et al., 1997; Clark & Brugge, 1995; Hynes, 1992; Ruoslahti, 1997). Fundamental cell behaviors, as for example, cell shape, size, migration, apoptosis and growth are influenced by mechanical and tensile properties of basement membranes. Choquet et al. (Choquet, Felsenfeld, & Sheetz, 1997) reported the capability of cells to sense restraining forces from basement membrane to strengthen cytoskeleton linkages, and that the strength of formed linkage depends on the rigidity and biochemical composition of ECM. Cell receptors act as transducers to transform the signals from basement membranes to nuclear or cellular region for the activation of gene or protein expression pathways, to control most of the activities of the cells in response to microenvironment (Chiquet, Matthisson, Koch, Tannheimer, & Chiquet-Ehrismann, 1996).

**Alteration of cell behavior through size, shape, and interconnectivity**

Traditionally cell shapes were controlled by attaching the cells into microbeads of different sizes (Chen et al., 1997) or by seeding cells on ECM coated substrates either varying the density of ECM or concentration of cell suspensions (Chen et al., 1997). These studies have suggested that the cell shape and size not only play an important role in survival of cells but also determine their proliferation rate and differentiation. With recent advancements in photolithography, microcontact printing, and surface
microtechnologies, cell shape, size and cell-cell interconnectivity have been controlled by different ligands, topography, mechanical or electrical cues. In accordance with previous studies, current studies have also shown that these factors play an important role in cellular behavior and fate (Guilak et al., 2009; Jiang, Bruzewicz, Wong, Piel, & Whitesides, 2005; Lauer et al., 2001; McBeath et al., 2004; Nelson & Chen, 2002; Thomas, Collier, Sfeir, & Healy, 2002; Yang, Co, & Ho, 2005a). In addition to the shape and size of the cells, cellular interconnectivity also plays an important role in cellular behavior and fate by facilitating juxtacrine signaling via cell-cell contact (Rosenthal et al., 2007). These signals are found to play an important role in stem cell differentiation and self-renewal (Javazon, Colter, Schwarz, & Prockop, 2001; Purpura, Aubin, & Zandstra, 2004; R. Y. Tsai & McKay, 2000; Zandstra, Le, Daley, Griffith, & Lauffenburger, 2000; Heasley, LE, 2001), tumor growth, and the creation of functional tissues in-vitro (Bhatia, Balis, Yarmush, & Toner, 1998).

**Size**

Chen *et al.* (Chen et al., 1997) conducted an experiment with human and bovine capillary cells on different sized fibronectin coated beads. Their result suggested that only 10% of the cells that were adhered to larger than 25μm beads went program cell death, but as the size of the beads were decreased, cells become more rounded and 60% of the cells underwent program cell death on 10 μm beads. Studies in different types of cells have proved that cell position, attachment, and nuclear and cellular spreading can be modulated by substrates containing different patterns of adhesive islands (Chen et al., 1997; Chen et al., 1998; Lauer et al., 2001; McBeath et al., 2004; Su, Jiang, Welsch, Whitesides, & So, 2007; Yang, Co, & Ho, 2005a) and thus affecting the cellular behavior. Apoptosis has been found to be decreased progressively in bovine and human capillary endothelial cells when they were seeded on islands of fibronectin ranging from 75 to 3000 μm² (Chen et al., 1997). Similarly, McBeath *et al.* (McBeath et al., 2004) seeded human mesenchymal stem cells (hMSC) on fibronectin islands of 1024 or 10000 μm² and observed the cell fate when cultured in mixed media that promote both
osteogenesis and adipogenesis. The cells which were seeded on small islands showed adipogenesis and on larger islands went osteogenic differentiation while mixed lineage were found on the mid-sized islands (Fig. 1-1).

![Image of cell shape and differentiation](image)

**Figure 1-1: Cell Shape Drives hMSC Commitment**

(A) Brightfield images of hMSCs plated onto small (1024 µm²) or culture on 1024 or 10000 µm² islands after 1 week in growth or mixed media. Lipids stain red; alkaline phosphatase stains blue. (B) Percentage differentiation of hMSCs plated onto 1024, 2025, 1000 µm² island after 1 week of culture in mixed media. Scale bar = 50 µm. (McBeath et al., 2004).

**Shape**

A link has also been made between stress gradients and proliferation rate. Nelson *et al.* (Nelson & Chen, 2002; Nelson et al., 2005) cultured Rat kidney epithelial cells on patterned square surfaces until confluent. BrdU incorporation was then used as a measure of proliferation rate and it was determined that the highest incorporation rates corresponded to locations where a finite elemental model predicted the cells would experience the most stress, namely the edges and corners. This correlation was confirmed by repeating the experiment on circles, rectangles and an undulating surface of tetrahedral pyramids.

Cells tend to grow to match the shape and size of the adhesive island as long as the area of the island is equal to or smaller than that of maximum spreading of the cell. Using this behavior, Chen *et al.* (Chen et al., 1997) showed that cells can be switched between growth and apoptosis by altering cell shape. Their study concluded that the
number of cells that underwent programed cell death was higher in circular or spherical shapes than that of planar non-patterned surfaces. Rounding of cells in spherical or circular shape induced cell death while spreading of the cells facilitated growth and proliferation. The effects of cell shape on cellular signaling has been found not to be limited to adhesion signaling (Guilak et al., 2009) since studies conducted in different cell types have suggested that cell shape may play an important role in their differentiation (Bhatia et al., 1998; Chen et al., 1998; Ingber, 1999; Mooney et al., 1992; Singhvi et al., 1994; Watt, Jordan, & O'Neill, 1988). Similarly, Guilak et al. and Manasek et al. (Guilak et al., 2009; Ingber, 1999; Manasek, Burnside, & Waterman, 1972) suggested that the change in cell shape may be one of the mechanisms that regulate myocardial development. Ingber et al. (Ingber, 1999) found that a change in cell shape mediated through the extracellular matrix, in part regulates the growth and differentiation of capillary endothelial cells. McBeath et al. (McBeath et al., 2004) reported the effect of cell shape on lineage commitment of MSCs showing that rounded cells tend to differentiate into adipocytes while the flattened cells show the commitment toward bone cells.

**Interconnectivity**

Interconnectivity has long been mentioned as a regulation mechanism of cell function but until recently methods had not been developed for a systemic study. Until recently the predominant method for studying cell interaction was using a co-culture model. Using this method co-culturing osteoblasts and mesenchymal stem cells has been shown to increase the rate of osteogenesis (M. T. Tsai, Lin, Huang, Lin, & Chang, 2011). In direct co-culture astrocytes were shown to attenuate neutrophil apoptosis and degranulation while enhancing their phagocytic ability (Xie et al., 2010). Intercellular communication has been shown to be necessary for the complete maturation of osteoblastic cells (Schiller, D'Ippolito, Balkan, Roos, & Howard, 2001). Direct cell contact has also been shown to induce transdifferentiation in mesenchymal stem cells into cardiomyocytes when placed in direct contact with neonatal cardiomyocyte (Yoon, Shim,
Ro, & Lim, 2005). Principally, research has focused on cadherins, notch, and gap junctions. Bloemen et al. (Bloemen, Schoenmaker, de Vries, & Everts, 2010) demonstrated that direct contact between fibroblasts and osteoclast progenitors up regulates osteoclastogenesis. Recent studies have begun to look at the mechanical ways that cell contact can affect the cytoskeleton (Nelson et al., 2005).

Gray et al. (Gray et al., 2008) developed an elegant way to study interconnectivity. They use dielectrophoresis to trap cells into an agarose gel with a controlled amount of cell to cell contact. They found that endothelial cells show a biphasic response to degree of interconnectivity with maximum proliferation at one contact. They explain that this makes sense in that cells with more than one contact would have contact inhibition versus cells with one contact, for instance at the edge of a wound, would be stimulated to proliferate more until they contacted other cells.

Using micropatterning of extracellular matrix proteins, Charest et al. (Joseph L. Charest and William P. King, 2008) showed that cell to cell contact of pre-keratinocytes modulated differentiation independent of island size or shape. This affect was linked to expression of E-cadherin. These new methods of cell patterning allow for increased resolution and systemic study of intercellular communication in the effect on cell function and fate.

**Techniques for chemical patterning, cell patterning, and cellular isolation**

Several techniques have been applied to create chemical patterns on the surface or to confine cells into predefined geometry. Irrespective of the techniques of patterning, their basis is photolithography. Initially, photolithography was developed for microelectronic circuits. Photolithography technique was first used in 1983 to investigate cellular behavior in confined geometry. Later Prime and Whitesides et al. (Prime & Whitesides, 1991) modified this technique and used to pattern biomolecules onto gold surfaces as self-assembled monolayers.
Cellular micropatterning mainly falls into three categories (described above) depending upon their strategies of cellular manipulation. Microcontact printing (µCP) is one of the oldest and most advanced techniques to pattern molecules onto the surface. Using this technique, pattern is transferred from an elastomeric stamp to a solid substrate by conformal contact. Cell repellent chemicals like PLL-g-PEG, alkanethiols, poly (OEGMA-co-MA), or a thin membrane of PDMS were used to backfill the protein-unpatterned portions (Chen et al., 1997; Frimat et al., 2010; Singhvi et al., 1994; Yang, Co, & Ho, 2005b). Switchable substrates (Jiang, Ferrigno, Mrksich, & Whitesides, 2003; Lahann et al., 2003; Jiang et al., 2003 have also been used to confine cells into microislands of patterned SAMs and then release cells from their confinements with dynamic control of interfacial properties that use conformational transitions of surface-confined molecules using an electrical potential. Elastomeric stencils have been used to spatially organize cells on various surfaces (Folch, Jo, Hurtado, Beebe, & Toner, 2000). This method uses a thin reusable membrane with holes on the surfaces before seeding the cells and then the membrane is manually peeled off after the attachment of the cells on the substrate. Retting et al. (Rettig & Folch, 2005) used PDMS micro-wells to trap individual cells. Recently, optical tweezers have been used to trap and actively manipulate biological cells (Birkbeck et al., 2003). Electrical tweezers via electrophoresis and dielectrophoresis (Gray et al., 2008; Voldman, 2005) have been used to control amount of cell to cell contact. Magnetic nano/micro-particles (Buyukhatipoglu, Chang, Sun, & Clyne, 2010) and piezoelectric spotting (Buzanska et al., 2010) are other alternative methods for cell pattering that employ one or more strategies to pattern the cells on various surfaces and materials (Rosenthal et al., 2007).

**Cellular confinement in designated geometry**

Long term maintenance of cells in pattern as well as their variability is essential to draw a concrete conclusion from a sequential study of cell behavior and their responses to
the microenvironment. Branch et al. (Branch, Wheeler, Brewer, & Leckband, 2000) reported confinement of rat hippocampal neuron cells up to a month period in serum free media, while the same group reported maintenance of cerebellar cell for up to 12 days in serum containing media (Branch et al., 2000; Branch, Wheeler, Brewer, & Leckband, 2001)(Fig. 1-2)

Surfaces are usually modified chemically before or after patterning of proteins for long term maintenance of cell integrity, viability and confinement within designated geometry. Most of the methods use the process of reduction of adsorbed serum components/proteins through appropriate chemical compositions to discourage cellular attachment or uncontrolled growth. Natural products like bovine serum albumin (BSA), agarose, and phospholipids show protein resistance (Nakanishi, Takarada, Yamaguchi, & Maeda, 2008). In addition to these natural products, other synthetic materials have also been used to render cellular confinements. Poly (ethylene glycol) (PEG) and its block copolymer with poly (L-lysine), poly-lactide, and polypropylene oxide (Pluronic) have been used successfully. Similarly, studies performed using polymer of phosphocholine, poly(2-methacrylethyl phosphorylcholine) (pMPC) have shown their effectiveness in resisting cell and protein attachments (Nakanishi et al., 2008). Yang et al. (Yang, Co, & Ho, 2005a), used random copolymers of oligo(ethylene glycol) methacrylate (OMEGA) and methacrylic acid (MA) on tissue culture plate to discourage attachment and absorption of proteins on the surface. These copolymers successfully confine human neuroblastoma cells in pattern for 5 days. Similarly, Frimat et al. (Frimat et al., 2010) used PDMS microfilms on tissue culture plates to confine differentiated human neuroblastoma cells in grid for the controlled formation of neuronal networks.

Hydrophobic molecules like alkylsalines have been studied to evaluate their effectiveness in discouraging protein and cell attachment during cell culture. Depending upon the nature of ECM secreted by cells and adsorbed protein conformation, these alkylsalines support or discourage cellular attachment (Britland, Clark, Connolly, & Moores, 1992; Kleinfeld, Kahler, & Hockberger, 1988).
Figure 1-2: Amount of biofilm adhering to stamped surfaces of PDL and flooded regions of PEG in culture over the duration of one month. Shown at each data point is the amount of biofilm (µg/cm²) adhered to PDL or PEG modified substrates. For PDL substrates, there is a dramatic increase in biofilm adhesion between 2 and 3 weeks, whereas only a nominal increase is observed on PEG modified substrates (Branch et al., 2000).

Polyethylene glycol (PEG) coated substrates have been widely used to minimize cells and protein attachment. It has been proven as one of the most effective polymers in minimizing effect of protein from serum-free or serum based media and cell secreted ECM in confining cells in patterns for weeks (Bernard et al., 2001; Branch et al., 2001; Gombotz, Wang, Horbett, & Hoffman, 1991; Prime & Whitesides, 1991; Singhvi et al., 1994; M. Zhang, Desai, & Ferrari, 1998).

Branch et al. (Branch et al., 2001) showed the amount of biofilms, proteins from media and ECM secreted from the cells, deposited on PEG immobilized for three weeks of culture is well below than required for cellular growth ~ 10 Å (Fig. 1-2). Their results
show that the cells start to grow their processes in PEG modified region after one month of culture.
Research Objectives

This study is divided in two different parts. First objective of this study is to establish live cell patterning techniques in the laboratory for the use of a systematic approach to investigate the effect of confined geometry, dimension and different extracellular matrix protein on the behavior and fate of stem cells or cell lines.

The second objective of this study is to relate the effect of ECM micropattern size on cellular behavior of human neuroblastoma cells for the effective regeneration of neurons and their function in neuronal regenerative medicine.

Organization of Thesis

This thesis will be divided into 5 chapters to put together all of the studies and findings in a logical order.

Chapter 1 summarized previous studies related to chemical surface patterning, effect of chemical patterning on cellular behavior in general, and the mechanism how cells sense chemical and mechanical signals to influence their behavior in-vivo and in-vitro. This chapter also briefly described the need and importance of effective methods of cell patterning in tissue regeneration and other many biotechnological applications. Lastly, this chapter summarized some of the currently used chemical manipulations to render cells into patterns and their confinement for longer period of time.

Chapter 2 will summarize first part of the study, establishing Soft Lithography, mainly Microcontact Printing, a powerful patterning technique for bimolecular and cellular patterning. This chapter will also contain some of the basic theories and principles of photolithography and their uses in tissue engineering, medical devices, cell biology and biotechnology. All of the experimental studies and results obtained during establishing of microcontact printing techniques in the lab will be included in this chapter along with short discussion and conclusion.
In Chapter 3, using our well established microcontact printing techniques, we will start second part of the study, which is to assess the effect of ECM micropatterns in the behavior of human neuroblastoma (SH-SY5Y) cells. This chapter will also include a short literature review of recent findings and studies related to neuronal cell patterning.

Chapter 4 will include experimental procedures, results, discussion and conclusion along with a short summary for behavioral study of human neuroblastoma cells on Collagen-I micropatterns.

Chapter 5 will be allocated for overall conclusion of these studies and recommend future studies to draw logical conclusions to move onto next level of behavioral study of neuronal cells or cells in general.
Chapter 2: Photolithography

Photolithography has long been used for microelectronic devices to pattern different chemicals and metals in circuits or microcircuits. In this method, a substrate, silicon wafer, where 3D micropatterns are to be printed is covered with a photosensitive polymeric material, the photoresist. When photoresist is exposed to radiation, usually UV, its chain structure is changed altering its solubility properties via crosslinking. Photolithography will etch or leave the exposed photosensitive chemical creating 3D microstructures on the surface. This substrate with arrays of microfeatures of photoresist on its surface is called a master. In cellular patterning, this master is used to transfer microfeatures in an elastomeric material to produced better results of contact between stamps and hard surfaces of cell culture substrates.

Arrays of 2D microfeatures are designed using computer software and printed on a glass surface, called a photomask. The features on the photomask either can be printed dark or clear depending upon the types of photoresist used. In photolithography, the photomask is placed on the top of substrate covered with a layer of photoresist and then UV light is illuminated enabling it to pass through selected areas. Depending upon the types of photoresist, the photoexposed part will be soluble or insoluble in developing solution.

In positive photoresist, exposed parts through clear areas of the mask will become soluble in developing solution and will be washed away with developer during development. In contrast, photoexposed parts of negative photoresist will be cross-linked to be insoluble, while the unexposed part will be soluble and washed away during development process. Therefore, it is very important to understand the type of photoresist and their properties before designing and printing a photomask.

Generally, in photolithography, exposing radiation falls under UV region having wave length of 365-436 nm. However, deep ultraviolet lights having wavelength of 193-248 nm and extreme ultraviolet radiation having wavelength of 10-14 nm have also been
used to obtained better resolution of the features that can be created using this method.
The resolution of the photolithography depends on the wavelength of radiation, distance
between photoresist and photomask, thickness and refractive index of photoresist.
Resolution obtained from such process can be calculated using following equation (Eeva
Lääniläinen, 2006).

\[ b_{\text{min}} = \frac{3}{2} \sqrt{\frac{\lambda}{n} \left( g + \frac{d}{2} \right)} \]

Where, \( b_{\text{min}} \) is smallest feature can be obtained, \( \lambda \) is wavelength of the UV radiation
used, \( n \) is refractive index of photoresist, \( g \) is the gap between photomask and resist layer
and \( d \) is thickness of the photoresist layer on silicon wafer.

**Soft lithography**

In topographical or chemical modification of the substrates for cellular patterning,
photolithography is followed by transferring 3D microstructures from a hard master to an
elastomeric stamp. This process is called soft lithography as coined by Prime and
Whitesides (Prime & Whitesides, 1991). Using this method, several stamps can be
molded from one mask reducing the use of cleanroom environment for fabrication of
master. This method is the basis of most of patterning techniques used in tissue
engineering, biotechnology or cell study assays.

**Materials for soft lithography**

Poly (dimethylsiloxane) (PDMS) elastomers (or silicone rubbers) have been used in
most of the soft lithography techniques. Similarly other elastomers, for example,
polyurethanes, polyimides, and cross-linked Novolac™ resins (a phenol formaldehyde
polymer), are also used in some studies (Younan Xia and George M. Whitesides, 1998).
PDMS offers a unique combination of properties due to inorganic siloxane backbone and organic methyl groups attached to its silicone molecules. They are liquid in room temperature due to their low glass transition temperature and can be converted into solid with an application of heat. Above glass transition temperature, molecules of PDMS move to a coordinated spaces due to thermal energy. This coordinated organization of molecules makes them soft and flexible upon solidification.

**Figure 2-1:** Polymerization of a mixture of PDMS prepolymer and curing upon application of heat (Eeva Lääniläinen, 2006).

PDMS is available in two different components as liquid prepolymer, usually called as base and liquid curing agent. Mixing and heating of these two components in appropriate ratio (w/w) produces rubbery and elastic solid. Stiffness of solid cross-linked polymer can be controlled by mixing these two components in different ratio. Mechanism of crosslinking between a prepolymer molecule and a curing agent under the application of heat is shown in Fig. 2-1.
Benefits of using an elastomeric stamp

Microscopy through PDMS stamp is easy as they are optically transparent. Their biocompatibility natures, inertness, non-toxic and resistant to many chemicals make them suitable in tissue engineering or biomaterials applications. Similarly, due to their low interfacial free energy (21.6 dyne/cm) (Younan Xia and George M. Whitesides, 1998) they can easily be released from a substrate or templates during casting and production of stamps or films.

Figure 2-2: Barrier tolerance of an elastomeric stamp and a hard stamp.

Printing of chemicals from a hard stamp to a hard substrate can be made difficult due to presence of a foreign particle between them. For hard substrate, when hard stamp is used, foreign particle will prevent contact between stamps and the transfer of pattern in entire area is disabled. But when an elastomeric stamp is used, this draw back can be greatly reduced as shown in Fig. 2-2 due to high tolerance of elastomeric stamps for foreign solid particles. Elastomeric stamps can stretch around foreign particles and will continue contacting other area enabling transfer of chemicals between substrates.

Most of the surfaces used for cellular patterning are microscopically rough. Transformation of continuous pattern on surfaces cannot be obtained through hard stamps as they are not capable of molding themselves as roughness of the surfaces. Soft stamps,
on the other hand, mold themselves according the roughness of patterning surface and make a conformal contact between surface and the stamp allowing a complete transfer of chemical or protein on the patterns as shown in Fig. 2-2, where $\lambda$ is amplitude of surface roughness (B. Michel A. Bernard A. Bietsch E. Delamarche M. Geissler D. Juncker H. Kind J.-P. Renault H. Rothuizen H. Schmid P. Schmidt-Winkel R. Stutz H. Wolf, 2001).


**Failure of an elastomeric stamp**

Despite of its great performance properties, PDMS also presents some technical problems in soft lithography. First, studies have shown that PDMS stamps along with
imprinted 3D microstructures shrink by ~ 1% during curing. Transformation of exact dimensions from the masters to stamps is made difficult when PDMS is used as molding agent. Similarly, cured PDMS stamps can swell with nonpolar organic solvent like toluene and hexane.

Similarly, due to softness of the elastomeric stamps, the aspect ratio \((h/l)\), as defined in Fig. 2-4, is limited greatly in soft lithography to achieve a proper transfer of chemicals from stamps to surfaces. According to Xia and Whitesides (Younan Xia and George M. Whitesides, 1998), obtainable range of values of dimensions of \(h\), \(d\) & \(l\) are 0.2-20, 0.5-200 and 0.5-200 µm. The aspect ratio \((h/l)\) of 0.2 to 2 is highly desirable for a stamp to work properly. If this aspect is too low or too high, stamps will fail as shown in Fig. 2-4.

Paring or collapse occurs when features buckle under their own weight due to lower gaps between features and longer heights of the features \((h>>d)\). Similarly, stamps will sag themselves under their own weight to touch substrate surface with entire region in between features if the distance between features is much larger than height of the features \((h<<d)\) as shown in Fig. 2-4 (Younan Xia and George M. Whitesides, 1998).

Figure 2-4: Diagram illustrating different failure events of an elastomeric stamp (Younan Xia and George M. Whitesides, 1998).
Microcontact Printing

Microcontact printing is a method of transferring inked materials form elastomeric stamps to substrate surfaces. This method is a continuation of photolithography and soft lithography. In lithography, a master with various arrays of microfeatures are created on a hard surface, which then is followed by soft lithography to mold an elastomeric stamp using the master. These stamps are used to ink the materials to be oriented or transferred in pattern to other substrates. A schematic diagram of lithography, photolithography and microcontact printing is illustrated in Fig. 2-5.

Master is fabricated exposing UV light to spin casted photoresist layer on silicon wafer as described above in introduction of photolithography. The master then is salinized with low surface energy agent, for example, Trichlorosilanes with CF2 groups or CFx molecules (Younan Xia and George M. Whitesides, 1998), to prevent attachment of photoresist materials on molds or PDMS surfaces during peeling.

Elastomeric stamps are fabricated by casting PDMS mixture on top of master and baking it for about 2 hours at 75 °C. Elastomeric stamps are peeled when they cool down to room temperature. These stamps are then cut into desired pieces for microcontact printing processes.

Stamps are inked with proteins or chemicals to be printed on the surface of substrate and let to adsorb on stamp surface. The inked stamps then are brought in contact with substrate for the transformation of inked materials.
Experimental section

In this section, all of the experimental procedures employed during the establishment of microcontact printing technique will be described. Also, this section will include information about materials and chemicals used for the experiments.

Materials and chemicals

FITC conjugated Collagen Type I- protein from bovine skin, Retinoic Acid, Hydroxypropylmethylcellulose (HPMC) were obtained from Sigma Aldrich (St. Louis, MO), Sylgard 184 silicone elastomer base & Sylgard 184 silicone elastomeric curing agent were purchased from Dow Corning corporation (Midland, MI). Photomask was obtained from HTA photmask (San Jose, CA), DAPI from Santa Cruz Biotechnology (Santa Cruz, CA), Dulbecco’s Modified Eagle Media (DMEM), penicicillin, trypsin/EDTA, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS) solution, rhodamine phalloidin from Invitrogen (Carlsbad, CA), PLL(20)-g[3.5]-PEG(2)-Rhodamin (TRITC) and PLL(20)-g[3.5]-PEG(2) were purchased from SuSoS
(Switzerland), Silicon wafers from Silicone Quest International Inc. (Reno, NV), Positive photoresist AZ P4620 and developer AZ 400K from AZ electronics (Somerville, NJ). Glass slides and coverslips and tissue culture plates were obtained from Fisher Scientific. Thin PDMS membranes were obtained from SMI (Saginaw, MI). Negative photoresist HNR 180 and WNRD negative resist developer were purchased from Fujifilm Electronic Materials.

**Mask Design**

Mask was designed using AutoCAD. Patterns of various complexities and wide range of dimensions were designed to test reproducibility and reliability of microcontact printing techniques. Another aspect of designing such patterns with different complexities was to closely mimic physiological conditions and interconnectivity of different types of cells in our body. Briefly, we designed parallel lines of 5-50 µm with a gap of 15-100 µm; nodes of 15-35 µm in diameter connected with 0, 2, 4, 6, 8 arms of 5-10 µm width. The reason behind using such dots and interconnected dots with variable arms is to manipulate the position of cells in nodes and their connection through processes following arm path.

**Master fabrication**

A 4” silicon wafer was cleaned with detergent and DI water and air dried. Clean wafers then were sonicated in acetone for 10 minutes followed by air dry and again sonication in ethanol for another 10 minutes. Two different types of photoresists having different viscosities were used to transfer microfeatures on silicone surface. Negative photoresist HNR-120 was spin casted at 2000 rpm for 20 seconds to form thin films (~ 3 µm). Positive photoresist AZ P4620 was spin casted onto the surface of wafer at 2000 rpm for 40 seconds to obtain thick films (~ 10 µm) of photoresist. The spin casted wafer was then soft backed at 95 ºC for 10 minutes. After cooling the wafer into room temperature, chrome photomask was placed on top of the wafer manually and transferred.
to Dymax 5000 Flood light curing system (DYMAX Corporation, Torrington, CT) for 3 seconds. Created wafer was developed in developer AZ 400K 1:4 DI water for 3 minutes and dried with the nitrogen gas. The resist master was silanized with chlorotrimethylsilane vapors overnight to prevent the transfer of photoresist to the PDMS during stamp fabrication. Method of master and stamps fabrication is illustrated in Fig. 2-5.

Figure 2-6: Diagram illustrating the fabrication of a PDMS stamp (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001).
Stamps Fabrication

A mixture of Sylgard 184 silicone elastomer base & Sylgard 184 silicone elastomeric curing agent was mixed well in a ratio of 10:1(w/w) and degased by centrifuging in 3000 rpm for 5 minutes. The degased mixture of PDMS and curing agent then casted onto the resist surface and cured for 2 hours in 70 °C oven and cooled in room temperature for 15 minutes before peeling out the 4 mm stamp off of resist master. As designed in chrome photomask, arrays of 5, 10, 20, 30, and 40 µm islands separated by 50 µm gaps; and dots of 15-35 µm diameter connected with 0, 2, 4, 6, 8 arms of 5-15 µm width were obtained separately in a square of 1 cm². Elastomeric stamps were then cut and stored in DI water until they were used.

Depending upon design of photomask or nature of photoresist (negative or positive), inverse or replica of microfeatures is printed on the silicone surface. If the inverse of the patterns are created on photoresist, only one molding of PDMS on master surface will create proper 3D features on stamps, but if patterns created on photoresist are not the inverse, then molding of PDMS on master substrate will create inverse of the patterns on stamp surface. Proper 3D features on the stamps can only be created by molding the PDMS again on surface of PDMS stamps.

Casting PDMS over PDMS is challenging since both PDMS layers adhere to each other. Proper demolding of two PDMS layers from each other was obtained by immersing the PDMS stamps (master) on a phosphate buffer solution (5 mM, pH 3) containing 0.1% (w/w) hydroxypropylcellulose (HPMC) for 10 minutes before molding another PDMS layer on its surface. This method is illustrated below in Fig. 2-7 (Gitlin, Schulze, & Belder, 2009).
Master and Stamps Characterization

As described earlier in this chapter, proper transformation of adsorbed protein from stamps to substrate surfaces depends on height as well as uniformity of features surface on the stamps. Depending upon the properties of photoresists, speed and time of spin casting, photoresist can be casted on silicon wafer surface in a wide range of thicknesses. We spin casted a negative photoresist HNR-120 and a positive photoresist AZ P4620 to create films of 3-10 µm height. Uniformity of stamp surface was achieved by adjusting UV exposure and developing time, and minimizing air bubbles during casting of
photoresists or PDMs mixture. Optical microscopy and 3D profilometry were used to characterize these parameters.

**Substrate preparation**

We used 25x75x1 mm plain microscope slides, 0.005” thick PDMS films of 4x4 cm and 35 mm tissue culture plates as substrate for micropatterning and cell culture.

**Surface modification of glass slides/coverslips and PDMS membrane**

Glass slides and PDMS membranes were cleaned with detergent, washed with DI water for several times and air dried. Air dried glass slides and PDMS membranes were sonicated in acetone for 10 minutes followed by air dry and again sonication in ethanol for another 10 minutes.

Glass slides were spin casted with Adhesion Promotor (AP) 6000 at 1000 rpm for 15 seconds and dried to facilitate adhesion of polystyrene on the surface. These glass sliders were further spin casted with 2% polystyrene at 1000 rpm for 15 seconds and dried.

PDMS membranes were fixed on the bottom of a circular stainless steel device with an elastic rubber band as shown in Fig. 2-8 to make patterning and cell culture process easier. Patterns were transferred in the middle circular area as seen in picture.
Figure 2-8: Thin PDMS membrane fixation to a metallic device. (A) Stainless steel device, (B) PDMS fixed in the device using an elastomeric rubber bad.

**Production of micropatterned surfaces**

In order to produce protein micropatterns for effective cell patterning, following steps were carried out.

**Chemicals and proteins**

A combination of different concentrations of Collagen-I, laminin, fibronectin and poly-d-lysine were used to ink the stamps. 100, 150, 200, 250 and 300 µg/mL of Collagen I and poly-d-lysine, 100 and 150 µg/mL of fibronectin and 50, 70, 90 and 100 µg/mL of laminin were used as inking materials for stamping process.

**Stamps and biosubstrate surface modification**

Before protein patterning, substrates were plasma treated for 10, 15, 20, 25 and 30 seconds to facilitate protein transformation from stamp surfaces. Similarly, stamps were treated in plasma for 20 sec to facilitate protein adsorption.
Microcontact printing

PDMS elastomeric stamps were cleaned with ethanol, dried in cell culture hood for 5 minutes and activated in the plasma chamber (Plasma- Preen® II, Plasmatic Systems Inc, North Brunswick, NJ) as described in section 2.5. Stamps were then inked with 50 µL protein solution (as described in section 2.5) and left to adsorb on stamp surface for 10, 20 and 30 minutes in dark. Remaining protein solution was aspirated with the pipette. Stamps were cleaned twice with DI water to remove unabsorbed protein from the surface and left to dry in the cell culture hood for 5 minutes. Dryness of the stamps was confirmed through the reflection of light. Clean 35 mm tissue culture plates were plasma activated in plasma chamber for 30 seconds in the flow of oxygen right before the stamping process. Stamps were carefully placed in contact with TCP surface as shown in Fig. 2-8. To assure the proper contact of the stamp and substrate, stamps were pushed gently with tweezers for 20 seconds (Fink et al., 2007) and applied uniform force of 4 glass slides from the top. To allow adequate transfer of adsorbed protein to the substrate, stamps were left in contact with surface for 10-60 minutes with an increment of 10 minutes, while in case of collagen-I, contact time was increased up to 90 minutes. Stamps were removed carefully and sonicated in water and ethanol for 15 minutes each before returning them to storage container for re-uses. The created micropatterned surfaces were either used immediately for further experiments or stored in PBS.

Passivation of non-printed areas through PLL-g-PEG backfilling

A 0.1 mg/mL of PLL (20)-g[3.5]-PEG(2)-Rhodamin (TRITC) solution was prepared in 10 mM HEPES buffer, pH 7.4. Micropatterned substrates stored in PBS were dried with nitrogen before backfilling with PEG, while dry substrates were used as without further treatments. A 100 µL PEG solution was poured per 1 cm² of printed substrate area and covered with a piece of dry and clean parafilm to minimize the amount
of PEG solution to use and make sure entire surface is covered with the solution. PEG solution was removed after 30 minutes and cleaned with PBS for 15 minutes to remove unattached PEG molecules.

**UV Sterilization of micropatterned substrates**

Protein micropatterned and PEG backfilled substrates were sterilized under UV in cell culture hood for an hour immediately before cell seeding.

**Cell culture**

Human Neuroblastoma cells (SH-SY5Y), mesenchymal stem cells (C3H10T(½)) and murine osteoblastic cells (MC3T3-E1) cells were seeded at 15000 cells/cm² in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penstrep, incubated at 37 °C and 5% CO₂ and allowed to attach. Media was supplemented with 0 or 10µg/mL of RA. Cells were checked under microscope every 30 minutes for 90 minutes until the satisfactory number of cells attach to protein patterns. Unattached cells were washed with PBS and cells were cultured for up to 14 days in complete media with change of fresh media every 3 days.

**Microscopy**

Protein patterns, attachment of cells and growth were recorded using Labomed optical microscope or Leica DMI 4000 B fluorescence microscope.
Results

Master and stamps

Transformation of 2D microfeatures from a photomask is illustrated in Fig. 2-8. Analysis of optical photographs conformed precise removal of exposed/unexposed photoresist from silicon wafer surface to replicate 2D features into 3D features. Grey lines in Fig. 2-9 A represent photoresist while lighter lines represent silicone surface.

After pouring and baking of PDMS on its surface for 2 hours, replica of features from master was obtained in PDMS stamps as shown in Fig. 2-9 B. Narrow lines represent groove while wider lines represent ridges of the transferred patterns.

![Image of transferred 3D microfeatures]

Figure 2-9: Optical images of transferred 3D microfeatures. (A) On silicon wafer surface. In this master dark lines represent photoresist. (B) An elastomeric PDMS stamp with 3D microfeatures transformed from master, where small rectangles represent grooves and big rectangles represent ridges (scale bar = 100 µm).

Reproducibility of protein patterns on various surfaces

To assess reproducibility of protein patterns, immuno-labeled ECM proteins were inked and transferred using different types of microfeatures. A fluorescence image of
transferred protein patterns (Fig. 2-10) on different surfaces confirms the efficacy and consistency of microcontact printing of protein using elastomeric stamps. Fig. 2-10 (A) represents 40 µm FITC conjugated collagen-I lanes separated by 50 µm gap. Similarly, Fig. 2-10 (B) and (C) represent interconnected grids. Immobilization of PEG on the surface is illustrated through Fig. 2-10 (D). Red surfaces obtained through fluorescence image confirms immobilization of TRITC conjugated PLL-g-PEG on the surfaces between collagen-I protein patterns (green).

Figure 2-10: Fluorescence images of transferred collagen-I conjugated with FITC ECM protein micropatterns on various surfaces. (A) parallel 40 µm lines separated by 50 µm gap on glass surface, (B) interconnected nodes of 35 µm diameter with 4 arms of 10 µm width on TCP, (C) interconnected nodes of 20 µm with 2 arms of 10 µm width on PDMS surface (D) 5 µm parallel lines separated by 50 µm gap backfilled with TRITC conjugated PLL-g-PEG on TCP surface (Scale bar = 50 µm).
To establish robust and flawless cellular patterning techniques, we investigated C3H10T(½) stem cells, MC3T3 osetoblastic cell line and SH-SY5Y human neuroblastoma cell for various combinations of ECM proteins and biosurfaces on different geometric confinements such as straight lines and interconnected nodes with 2 arms or 4 arms. All three cell lines were found to have quite different attachment properties. SH-SY5Y cells showed the lowest attachment when compared with other two cell lines, while MC3T3 showed the best patterning results.

Figure 2-11: Reproducible cellular patterns on different surfaces. (A) C3H10T(½) cells attached to collagen-I on glass surface (B) MC3T3 cells on fibronectin on glass surface (C) MC3T3 on PDL on glass surface (D) MC3T3 on collagen-I on TCP surface (E) MC3T3 on fibronectin on TCP surface (F) C3H10T(½) on collagen-I on TCP
Cell patterning capability varied with the basal substrate type on which proteins are patterned. All of three cell lines had a very good attachment on TCP-based patterns. MC3T3 and C3H10T (½) cells preferred PDMS and SH-SY5Y showed a satisfactory attachment on PDMS. Even though all of the three cell lines attach to glass slide and coverslip surfaces, number of attached cells was very low on glass-based patterns even after 4 hours of seeding time.

For most of the cases, cells remained well confined into geometry for up to 9 days with some interconnection among neighboring cells from adjacent patterns. Detachment and tearing of entire row/column of cell patterns was observed on glass and coverslips after few days of culturing.

**Parameter optimization**

Based on qualitative results obtained from optical and fluorescence images of protein and cell patterns, we have optimized various parameters, for example, concentration of ECM proteins, plasma treatment and hydrophobicity of stamps, adsorption time and amount of protein solution required to ink, printing time and force applied to the stamps for successful reproduction of protein patterns on various biosurfaces as shown in Table 1.

Use of negative photoresists for patterns printed on clear area of photomask requires a second molding to create upright patterns on PDMS stamps, while this photoresist can be used to transfer replica of patterns from photomask to silicon wafer surfaces directly if the printed patterns on the photomask are dark. Positive photoresist behave contrary to negative photoresists.
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Substrates</th>
<th>Concentration (µg/mL)</th>
<th>Protein Sol(µL/cm²)</th>
<th>Plasma Treatment (sec)</th>
<th>Inking Time (minutes)</th>
<th>Printing Time (minutes)</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>PDMS</td>
<td>250-300</td>
<td>70</td>
<td>20</td>
<td>30</td>
<td>60-90</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td></td>
<td>Glass Slides</td>
<td>250</td>
<td>50</td>
<td>30</td>
<td>30</td>
<td>60</td>
<td>4 glass slides</td>
</tr>
<tr>
<td></td>
<td>TCP</td>
<td>200</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td>Poly-D-Lysine</td>
<td>PDMS</td>
<td>250</td>
<td>70</td>
<td>20</td>
<td>30</td>
<td>50</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td></td>
<td>Glass Slides</td>
<td>250</td>
<td>50</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>4 glass slides</td>
</tr>
<tr>
<td></td>
<td>TCP</td>
<td>200</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>PDMS</td>
<td>150</td>
<td>40</td>
<td>10</td>
<td>10</td>
<td>30</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td></td>
<td>Glass Slides</td>
<td>100</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>4 glass slides</td>
</tr>
<tr>
<td></td>
<td>TCP</td>
<td>100</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td>Laminin</td>
<td>PDMS</td>
<td>90-100</td>
<td>40</td>
<td>10</td>
<td>10</td>
<td>30</td>
<td>Gentle tweezers tapping</td>
</tr>
<tr>
<td></td>
<td>Glass Slides</td>
<td>70-90</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>4 glass slides</td>
</tr>
<tr>
<td></td>
<td>TCP</td>
<td>50</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>Gentle tweezers tapping</td>
</tr>
</tbody>
</table>

Table 1: optimized parameters for microcontact printing.
Based on the design of our photomask, we preferred positive photoresist to eliminate second step of PDMS to PDMS molding to create features on the stamps. Moreover, obtaining the optimum aspect ratio \( h/l \) for created microfeatures on stamps as described in the introduction was difficult for negative photoresist due to limitation on thickness of the photoresist layer that can be casted on silicone surface. On the other hand, AZ P4620 provided flexible height of casted resist to meet requirement of aspect ratio enabling better printing results as it can be casted up to 40 µm thick layer on silicon wafer.

**Discussion**

Transformation of protein from stamps created using HNR-180 photoresist did not show satisfactory results in terms of well separated transferred features. This is probably due to the aspect ratio of features on stamps. The highest thickness of photoresist layer that can be casted on silicon wafer is \(~3\) µm, but our features ranged from 5-40 µm in width. The aspect ratio for larger features was beyond suggested range which is 0.2-2. Due to very low aspect ratio, stamps fail to hold themselves and sagged touching the substrate surface with entire area transferring protein. On the other hand, AZP 4620 can be casted in 10-40 µm thickness allowing us to create workable stamps a great range of pattern sizes. For our design of 5-40 µm width features, 10 µm heights perfectly hold true within the suggested aspect ratio for good stamps.

Stamps with proper aspect ratios used for protein patterning produced printed patterns of all of four different extracellular matrix proteins on three different surfaces. However, all of 3 surfaces behaved differently during microcontact printing of ECMs. TCP and PDMS were relatively better than glass surfaces in terms of accepting adsorbed protein from stamps and contact between two surfaces. Four proteins also behaved differently in terms of adsorption and transformation of the protein during microcontact printing. This is due to variation on surface chemistries among surfaces.
Depending upon the nature of ECM proteins, their rigidity and biochemical composition, adsorption time and transformation efficiency of adsorbed protein can be varied. For example, our data have showed that Laminin and Fibronectin can be adsorbed as short as 10 minutes of inking while collagen-I and Poly-D-Lysine take about 30 minutes. Similarly, 5 minutes of contact between substrate surface and microstamps along with application of gentle force from tweezers can transform satisfactory amount of Laminin and Fibronectin ECM proteins for most of the surfaces used. However, Collagen-I and Poly-D-Lysine took an hour for the similar amount of protein to be transferred.

TCP, glass and PDMS surfaces supported all four ECM adsorbed protein transformation from stamps. Fluorescence image analysis showed the capability of transformation of > 70 % of patterns from the stamps. Corners and edges contained some of discontinued or overlapped transferred patterns. While in some cases, patterns were missing in the center probably due to lack of proper contact between stamp and surface.

Adherence of PLL-g-PEG was significantly dependent upon the surfaces. PDMS films turned out to be least favorable surfaces for its adhesion. Additionally, glass slides and glass cover slips were modified with polystyrene to discourage attachment and migration of cells on background region. However, their effectiveness in blocking cell and protein attachment was not effective. PEG preferred TCP out of three surfaces and transferred PEG could be conformed from fluorescence images as demonstrated in Fig. 2-10 D.

ECM proteins facilitate attachment of cells. As seen in Fig. 2-11, patterning of various combinations of proteins, substrate surfaces and cell types is possible through microcontact printing. For substrate surfaces without any cell or protein resistant, maintaining of cells on pattern for longer time is difficult as cells proliferate and migrate to neighboring areas covering the entire available space for them to move or grow. Cell and protein repellents, such as PLL-g-PEG, restrict cells only on patterns discouraging their migration and communication with other cells for neighboring patterns. Studies
have shown that cells can be confined within the pattern for up to 26 days (Branch et al., 2000; Corey et al., 2010) with the application of these cell repellents, but in our study we noticed cells starting to migrate to neighboring area on day 13. This is probably due to deposition of ECM proteins from cells and supplied media on the PEG surface or degradation of PEG layer from the surface due to continuous exchange and shaking of media during imaging.

**Conclusion**

Photolithography and soft lithography have been used for decades in cellular patterning. Due to different behavior of substrate surfaces, ECM proteins and cell lines, cell patterning in general may be unsuccessful for specific combination of protein, surface and cell line. Therefore, optimizing all of the variables during techniques could produce better results on protein and cell patterning.

Using optimized parameters, we have successfully patterned 3 different cell lines on 3 different surfaces using 4 different extracellular matrix proteins. Patterning of protein and cells varies according to surface chemistries of the substrate and stamps, nature of cell, and biochemical properties of ECM proteins.

Long term cellular confinement without application of cell and protein deterrent is impossible. Application of these repellents can hold cells within defined geometry for weeks allowing their better organization and interconnectivity. Studies conducted on such highly organized cell could produce better understanding of cells and their behavior enabling effective regeneration of tissues.
Chapter 3: Human Neuroblastoma Cell Patterning for Behavioral Studies

Introduction

Thousands of neurological disorders are reported every year due to loss of functional neuronal circuits or effective regeneration failure within body. According to American Cancer Society (6/15/2011), neuroblastoma cancer accounts for 7% of all infant cancers. Traumatic brain injuries affect hundreds of thousands of people every year in the United States alone and more prevalent in the US army due war in Iraq and Afghanistan. Alzheimer's disease, Parkinson's disease, and multiple sclerosis are other prominent neurological disorders seen among a big number of people. Similarly, neurotoxic chemicals released during war, fire, explosion or natural calamities also account for a big number of neuronal disorders cases around the world.

Proper organization of neuronal cells and their interconnectivity in-vitro mimicking physiological condition of human body may enable us to understand the mechanisms behind all of these disorders at cellular level. This method enables us to study cell behaviors and abnormalities such as cell adhesion, proliferation, apoptosis, differentiation and migration of neuronal cells. These behaviors are central to pathophysiology of many neuronal disorders, and understating them is a key towards successful addressing of human neurological disorders.

Similarly, there is an urgent and immediate need of an effective screening of neurotoxic chemicals that possess health risk to human nervous system. Current lengthy methods use rodents and other primates to assess the risk of chemical to neurons. These methods are ineffective as they take several days to provide data. Therefore, proper organization of neuronal cells mimicking animal models and in-vivo human conditions is in strong need for better screening approaches of such toxic chemicals.
Literature review

Studies have shown that in-vitro culture of neuronal cells can be used as an effective method to assess their development. However, these cultures in homogeneous surface cannot provide the complexities that neuron face within our body. Proper spatial organization of neuronal cells and their interconnectivity on synthetic substrates would mimic in-vivo environment to allow better understating of cellular behavior, and their development. Understanding of these behaviors in cellular level can provide an effective method of tissue regeneration and restoration of their function.

Several attempts have been made to restore neuronal function after injury. Application of growth factors (Vincent & Feldman, 2002), stem cell differentiation to neuronal cells (Horner & Gage, 2000) and electrical stimulation of injured neuron cells (Grill et al., 2001; Peckham et al., 2002). All of these processes introduce cells with external environment, the biomaterials surface and synthetic or cell secreted extracellular matrix proteins. These surfaces and ECM can be applied into various combinations, and each combination can produce different effect on cellular behavior and interaction of cells with these external environments (Joseph M. Corey and Eva L. Feldman, 2003) and can serve as a means to regenerate neuronal cell in-vitro (Condic & Lemons, 2002).

As described in chapter 1, biomaterial surfaces and extracellular adhesion molecules affect adhesion, migration, proliferation, survival, growth, apoptosis, neurite outgrowth, axonal regeneration and polarity of neuronal cells or cells in general (Blesch, Lu, & Tuszynski, 2002; Buzanska et al., 2010; Chen et al., 1997; Chen et al., 1998; Chen, Alonso, Ostuni, Whitesides, & Ingber, 2003; Corey et al., 1997; Frimat et al., 2010; Klein, Scholl, & Maelicke, 1999; Lauer et al., 2001; Letourneau, Condic, & Snow, 1994; McBeath et al., 2004; Morin et al., 2006; Nakajima et al., 2007; Thery et al., 2005; Vincent & Feldman, 2002; Yang, Co, & Ho, 2005a; J. Zhang et al., 2006). Based on these results, several efforts have been made to understand the interaction of ECM and biomaterials with neuronal cells using user-defined surfaces and biochemical factors such
as ECM. One of highly studied methods in understating such relation is 2D patterning of neuronal cells using ECM molecules in a fixed geometry to enhance adhesion, control cell-cell connectivity, migration, proliferation, apoptosis and differentiation.

Most of the studies conducted on neuronal cell patterning are related to neurites and their controlled growth, and nodal compliance (Branch, Corey, Weyhenmeyer, Brewer, & Wheeler, 1998; Corey, Wheeler, & Brewer, 1991; Frimat et al., 2010; Klein et al., 1999; Lauer et al., 2001; Morin et al., 2006; Scholl et al., 2000; Suzuki, Sugio, Jimbo, & Yasuda, 2005; Wheeler, Corey, Brewer, & Branch, 1999). Recently, studies have shown that nodal compliance in adhesive patterns produces more controlled and directed neurite growth in the culture (Frimat et al., 2010; Lauer et al., 2001; Yang, Co, & Ho, 2005a). These studies have shown that cell spreading is greatly affected by the size of adhesive islands decreasing nodal compliance with increasing width of the cellular adhesive cues. Interestingly, Yang et al. (Yang, Co, & Ho, 2005a) found that the neurite growth can be enhanced by patterning cells. His study showed that length of neurites on patterned surfaces are significantly longer than that of unpatterned cells, and better neurite growth can be obtained when these cells are patterned in smaller island like 5 µm.

Confinement of neuronal cells also affects their organization, interconnectivity, cellular and nuclear area. Recently, studies are conducted not only to confine neuronal cells into patterns (Corey et al., 2010) but also asses their behavior and growth in different geometry (Yang, Co, & Ho, 2005a). However, there have been very limited understandings on the effects of combination of micropattern size and specific ECM proteins used for patterning on neuronal cell behavior. In this study, we have examined the effect of Collagen-I ECM protein on human neuroblastoma (SH-SY5Y) cell morphology and behavior when patterned separately using 5, 10, 20, 30 and 40 µm wide straight lines separated by a 50 µm gap.
Chapter 4 : Experimental Section

Materials and methods

Glass coverslips/slides and TCP have been widely used for cell culture and patterning purposes. Glass slides/coverslips are relatively cheap and excellent in microscopy. As mentioned in earlier section, glass surfaces with or without surface modification before PEG flooding did not show a satisfactory result in terms of PEG attachment, protein pattern transfer, cellular confinement or stability of protein/cell pattern. Also, handling and live cell microscopy of coverslips/glass slide was inconvenient as they are prone to physical damages.

Even though there are not many studies involving cell patterning in PDMS films, we considered it as a promising candidate as it is cheap, easily available and easily producible in laboratory. Another reason to pursue PDMS in this study is to use pattern cells for mechanical testing, as for example, uniform uniaxial stretching of cells. PDMS supported excellent cell attachment when there was enough amount of protein transferred on the pattern and also well confined cells to assigned geometry (due to its hydrophobicity) despite of poor PEG immobilization. Air bubbles in some regions made discontinuations in protein patterns. Moreover, due to its stretchable property, cells can experience mechanical stretching during microscopy, media change or mounting the cells for further uses. These unwanted variables during experiments might produce inaccurate results in behavioral study of the cells. Therefore, we excluded PDMS and Glass surfaces in assessing neuronal cell behavior under patterning.

Our studies show there are some differences among ECM proteins in their adsorption on stamp surface, transfer efficacy and cellular attachment. In this study, we used collagen-I ECM proteins as it has shown excellent cell attachment and transfer on TCP surfaces.

All of the other materials were used as described in chapter 2.
Photolithography and stamp fabrication

Detailed methods of photolithography and stamp fabrication process were described in chapter 2. In summary, 10 µm AZP4620 photoresist layer was spin casted on a 4” silicon wafer. After soft baking, photoresist layer was exposed to UV light for 3 seconds sandwiched between photomask and wafer. The wafer was then developed to produce master with replica of features. Threes replica were again replicated by pouring a PDMS mixture and baking it for 2 hours at 75 °C. Stamps were cut into 1 cm² area and stored in DI water in a 50 mL centrifuge tube.

Microcontact printing

Microcontact printing is explained in Chapter two. For this study we used parallel line of 5, 10, 20, 30 and 40 µm width separated by a 50 µm gap. 250 µg/mL Collagen-I ECM protein was used as inking material. 50 µL of protein solution was inked per square cm of stamp and left to adsorb in dark for 30 minutes. Non-adsorbed protein solution was washed once with PBS and once with DI water. Stamps were dried completely and brought in contact with TCP surface for 30 minutes with application of occasional tweezers tapping.

Cell culture

Using complete media, 15000 SH-SY5Y (P7) cells were seeded per cm² of patterned area as described below.

Twenty different 35 mm TCP were micropatterned and divided into four groups. The first group of 5, 10, 20, 30 and 40 µm wide lines contained no PEG backfilling and the media supplemented in this group did not contain any retinoic acid (RA). Five TCP with 5, 10, 20, 30 and 40 µm micropatterned lines separately were not backfilled with
PEG but the media was supplemented with 10µg/mL RA. Similarly, 3rd and 4th groups were backfilled with PEG but only group 4 was supplemented with 10µg/mL RA in the media.

Cells were incubated on 37 ºC and 5 % CO₂ and checked at every 30 minutes to observe attachment of cells. After 90 minutes, unattached cells were washed with PBS. Cellular behavior was recorded by live cell imaging under microscope every other day for 7 days.

**Fluorescence imaging**

Patterned SH-SY5Y cells were fixed on day 7 with 4 % paraformaldehyde for 10 minutes and permeablized with 0.1% Triton X-100. Actin cytoskeleton was stained with 5 µL/mL rhodamine phalloidin in PBS for 20 minutes. Nuclei were stained with 2µL/mL DAPI in PBS for 2 minutes. Cells were mounted using mounting liquid and glass coverslips. Images were taken using fluorescence microscope.

**Measurement of neuronal cell behavioral**

**Nuclear orientation**

Nuclear orientation was measured by using ImageJ angular measurement. In this measurement, vertical line parallel to protein patterns were referenced as 0 degree of orientation or perfectly aligning references. Right or left deviation of nucleus from reference line was considered as an orientation of that particular nucleus as shown in Fig. 4-1. For each case, at least 100 nuclei were used to determine the percentage of aligned cells along the patterns.
Figure 4-1: Schematic diagram of nuclear orientation measurement. Straight line parallel to protein pattern was regarded as zero degree, and the right/left deviation from the reference line was measured as the orientation of that particular nucleus.

**Cellular area**

Fluorescence images were analyzed using ImageJ image analyzing software to measure average cellular area of cells patterned in 300x300 µm² of substrate surface. Fluorescence images were subjected to enhanced contrast to clearly differentiate background Fig. 4-2 (A) and cellular area (object). Enhanced images were then subjected to threshold color for precise exclusion of background noise (B). Adjustment of threshold color further provided the exclusion of gap in between cells or group of cells as shown in (C). The cellular spreading area then was selected carefully (D & E) and the area within the curves were measured. This measurement gave a total area of the cells within 300x300 µm².
Figure 4-2: measurement of cellular area using imageJ image analyzing software. (A) Original RGB image obtained with fluorescence microscope, (B) after enhanced contrast among the colors, (C) after application of threshold, (D) after adjustment of threshold colors, (E) after selection of cellular area, and (F) selection of only cell area excluding nuclear areas.

By analyzing the same picture with only nuclear (DAPI) staining, total number of cells attached to specific area was obtained by counting total number of nuclei spotted in that area. Average cell area then was calculated by dividing the total cellular spreading area by total number of nuclei (cells). At least four different pictures were analyzed for each condition.

**Nuclear area**

Nuclear area was also measured by using the same method as cellular area, except in this case particle analysis was applied after threshold and selection. DAPI staining showed that nuclei do not overlap themselves as seen in Fig. 4-3(B). Using these figures, number of total nuclei in that area can be calculated because of the spaces among them. Particle analysis could create more precise measurement excluding those gaps between nuclei.
Figure 4-3: particle analysis fluorescence images to calculate nuclear area using imajeJ image analyzing software. (A) Original image of nuclei obtained with fluorescence microscope of DAPI staining, (B) after enhanced contrast among colors, (C) after application of threshold, (D) after adjustment of threshold colors, (E) after selection of cellular area, and (F) outline with numbered areas obtained after particle analysis of nuclei.

Neurite growth

Neurite extensions of cells were measured by using NeuronJ add-on of ImageJ analyzing software. Cells in the area of 300x300 µm² were analyzed to be consistent through the study.

Neurite extension was traced and length of each neurite was measured (Fig. 4-4). By using this method exact path of each neurite can be traced out whether it’s straight or zigzag. To be consistent among the measurements, cellular extensions were considered as neurite when their width was less of equal to 4.5 µm.
Figure 4-4: Neurite extension measurement using NeuronJ add-on of ImageJ image analyzing software.

Data analysis

All experiments were repeated at least 4 times. Mean and standard deviation for each set of data were calculated and statistical significance was determined by ANOVA using Student-Newman-Keuls test.
Results

Reproducibility of protein patterns and PLL-g-PEG backfilling

Figure 4-5: Fluorescence images of ECM protein patterns and PLL-g-PEG backfilling on tissue culture plate surface. FITC conjugated collagen-I lanes (A) 5 µm (B) 20 µm and (C) 5 µm FITC conjugated collagen-I lanes backfilled with TXRD conjugated PLL-g-PEG (Scale bar = 50 µm).

Fluorescence images of patterns of Collagen-I conjugated with FITC on TCP are shown above in Fig. 3-1. In Fig. 4-5 A and B, dark green lines represent protein patterns while black background is the bare TCP surface. Similarly in Fig. 4-5 C, the background is flooded with PLL-g-PEG conjugated TRITC. Our results show approximately 70% of the area can repeatedly be covered with protein patterns except some edges and corners.

Patterned neuronal cell response

Cellular adhesion and proliferation

To evaluate cellular adhesion on ECM patterns, SH-SY5Y cells were seeded at 15000 cells/cm² area of micropatterned surface for 90 minutes with or without PEG backfilling. Cells were then washed with PBS and incubated in 37 °C and 5 % CO₂ using complete media.
Initially, attachment of cells on Collagen-I lanes were confirmed under microscope quickly but gently to avoid detachment of freshly attached cells due to media movement during optical microscopy. Moreover, exposing such attached cell uncontrolled environment could create adverse effect on cells and their behavior. Microscopy of these samples confirmed attachment of cells on pattern and areas in between patterns collagen ECM.

Optical images were taken the next day (day 1) to further evaluate attachment and proliferation of cells as shown in Fig. 4-6 and 4-7. Without PEG backfilling, cells did not follow patterns of 5 μm lanes at all as shown in Fig. 4-6 (A). Without PEG backfilling, cells were not only attached to 10, 20, 30 and 40 μm collagen patterns but were also

Figure 4-6: SH-SY5Y cells on collagen-I ECM patterns on day 1 without any backfilling. (A) 5 μm, (B) 10 μm, (C) 20 μm, (D) 30 μm, (E) 40 μm, and (F) control (scale bar = 50 μm).
attached to surfaces beyond patterns. Also in these conditions, cells from one ECM lane grew to neighboring lanes (Fig. 4-6 B, C, D & E).

Figure 4-7: SH-SY5Y cells on collagen-I ECM patterns on day 1 with PLL-g-PEG backfilling. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D) 30 µm, and (E) 40 µm proteins lanes separated by 50 µm gap (scale bar = 50 µm).

Distinct patterns of cells were observed in 5 µm lane when PEG was immobilized to backfill surfaces as shown in Fig. 4-7 (A). Application of PEG confined all of seeded and attached cells to assigned geometry in all cases. For smaller patterns like 5 and 10 µm, single cell occupy the width of patterns (Fig. 4-6 B, 4-7 A and B), whereas multiple cells occupy larger islands of ECM lanes (Fig. 4-6 C, D & E, and Fig. 4-7 C, D & E).
Effect of PLL-g-PEG on cellular confinement

To evaluate the efficacy of PEG to render cells and their processes within predefined geometry, human neuroblastoma cells were seeded on with or without PEG immobilized surfaces. Media was either supplemented with 0 or 10 µg/mL retinoic acids for 7 days. Optical images were taken on day 7.

![Fig. 4-8: SH-SY5Y cells on collagen-I ECM pattern day 7 without any backfilling and no RA. (A) 5µm, (B) 10 µm, (C) 20 µm, (D)30 µm, (E) 40 µm, and (F) control (scale bar= 50 µm).](image)

PEG confined cells and their attachment for both cases of RA supplemented or non-supplemented media (Fig. 4-10 and 4-11). Continuous supplement of RA for seven days induces differentiation of neuroblastoma cells and causes significant increase in neurite extension (Fig. 4-8, 9, 10, 11). These neurites extensions in PEG backfilled surfaces also followed the path of protein pattern maintaining very well organized cell patterns for seven days (Fig. 4-10 A, B, and 4-11 A, B).
Figure 4-9: SH-SY5Y cells on collagen-I ECM pattern on day 7 without any backfilling and with 10 µM RA (A) 5µm, (B) 10 µm, (C) 20 µm, (D)30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm).

Cell pattern on surfaces without any cell repellent backfilling did not hold perfect patterns in both cases of RA (Fig. 4-8, 4-9). Cells started to migrate and proliferate to gaps on day 4 and kept growing until day 7 completely transpassing patterns in 30 and 40 µm wide lines (Fig. 4-8 D, E, and 4-9 D, E). For smaller island of 10 and 20 µm, some cells still were intact on the patterns even though they were growing randomly connecting with cells on close patterns (Fig. 3-8 B, C, and 3-9 B,C).
Figure 4-10: SH-SY5Y cells on collagen-I ECM pattern on day 7 with backfilling but without RA. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D) 30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm).

Figure 4-11: Optical images of SH-SY5Y cells on collagen-I ECM pattern on day 7 with backfilling and with 10 µM RA. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D) 30 µm, (E) 40 µm, and (F) control (scale bar = 50 µm).
Effect of micropattern size on cellular morphology

Pattern of human neuroblastoma cells could not be maintained on patterns without PEG backfilling for longer time as they grew and migrated to surrounding areas. Confluence of cells on these micropatterned regions was seen equal or greater than cells on non-patterned control group. Therefore, samples prepared without using PEG backfilling were removed from further analysis as they could not provide user defined geometry for cells to grow and would not produce concrete conclusion on cellular behavior from their analysis.

Figure 4-12: Fluorescence images of SH-SY5Y cells on collagen-I ECM pattern day 7 without RA. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D) 30 µm, (E) 40 µm, and (F) control. Red = actin cytoskeleton, blue = nucleus (scale bar = 50 µm).
Immunostained fluorescence images of cells in pattern were analyzed qualitatively. Actin cytoskeletons of cells were stained with rhodamine phalloidin and nuclei were stained with DAPI for better visualization and contrasts.

Very low number of cells was attached on 10 and 20 µm ECM lanes producing discontinuous patterns (Fig. 4-12 A, B, and 4-13 A, B). In these narrow lanes, only one cell prominently cover the entire width and connected with neighboring cells in the same pattern with an extension of long neurite along the protein pattern. Even though differentiated neuronal cells usually have neurite extensions in multiple directions, cells in these two lane widths only had 2 major neurites to connect with immediate two cells in the same pattern. Cells tend to grow slightly beyond the width of protein patterns in such narrow lanes. Cells were highly organized in terms of their orientation toward the main axis of ECM islands for 5 and 10 µm.

Figure 4-13: Fluorescence images of SH-SY5Y cells on collagen-I ECM pattern on day 7 with 10 µM RA. (A) 5 µm, (B) 10 µm, (C) 20 µm, (D) 30 µm, (E) 40 µm, (F) control. Red=actin cytoskeleton, blue= nucleus (scale bar = 50 µm).
ECM islands of 20, 30 and 40 µm contained multiple cells to cover entire width (Fig. 4-12 C, D, F; and Fig. 4-13C, D, F). Due to highly compact and overlapping of cells in these patterns, distinct neurite extensions were not visible in contrast to 5 and 10 µm lanes. Similarly, cells orientated themselves randomly. In accordance with narrow lanes, cells in these patterns grew few microns beyond their defined geometry and no random extensions of neurites among pattern were seen for at least seven days.

**Quantified cellular behavior**

Means and standard deviations were calculated for each set of data, and statistical significance among different sets was calculated by using ANOVA. Cellular and nuclear spreading area, extension of neurites, and orientation of nuclei were varied depending upon the width of ECM patterns. These variations were also seen in samples that were supplemented with or without retinoic acid.

![Figure 4-14: Analysis of cellular and nuclear spreading area for SH-SY5Y neuroblastoma cells on day 7 without supplement of retinoic acid.](image-url)
Fig. 4-14 represents variation of cellular and nuclear area among cells that were grown in different ECM pattern width without retinoic acid. Nuclear spreading and cellular spreading on 5 and 10 µm ECM lanes were significantly smaller (p<0.001) than nuclear and cellular spreading on control, 20, 30 and 40 µm lanes. Nuclear and cellular area in 20, 30 and 40 µm were statistically insignificant (p>0.05) with control group and among each other. Also, spreading of nuclei and cells on 5 and 10 µm was statistically insignificant (p>0.05). These results suggest that cellular and nuclear spreading and their growth are greatly influenced by pattern sizes used in cellular patterning.

Figure 4-15: Analysis of cellular and nuclear spreading area for SH-SY5Y neuroblastoma cells at day 7 with supplement of retinoic acid.

Graph on Fig. 4-15 represents cellular and nuclear spreading area on ECM lanes width with RA supplementation. Analysis of these data show exactly same trends for
nuclear area as mentioned above. Spreading of nuclei area on 5 and 10 μm is significantly smaller (p<0.001) than that of control, 20, 30 and 40 μm ECM lanes, and spreading of nuclei among control, 20, 30 and 40 μm is statically insignificant (p>0.05). On the other hand, cellular spreading in this case was independent of ECM width and is statistically insignificant among all groups.

Figure 4-16: Analysis of nuclear orientation for SH-SY5Y neuroblastoma cells on day 7 without retinoic acid.

Orientation of cells is influenced by orientation of their nuclei. To measure orientation of nuclei, we referenced a parallel line to protein patterns as 0° (prefect orientation) and deviation of left or right from this reference line was used as the orientation of nuclei.

Orientation of nuclei also varied with the width of ECM patterns used. Graph in Fig. 4-16 shows, well above 90 % of SH-SY5Y cells nuclei after 7 days of culture on 5
and 10 µm collagen lanes had perfect or nearly perfect (<10°) orientation along protein patterns, e.g., 100% of nuclei in these two patterns were aligned within 20° of deviation from protein patterns. Higher number of cells showed increase in deviation due to increase in pattern width. In comparison among pattern widths, 5 and 10 µm contained less deviated cells, while 20 µm showed satisfactory result in nuclei orientation by orientating more than 80% cells within 20°. The 30 and 40 µm lines had the most randomly oriented cells in this group.

**Figure 4-17: Analysis of nuclear orientation for SH-SY5Y neuroblastoma cells on day 7 with 10 µg/mL retinoic acid.**

In comparison with undifferentiated cells, nuclear orientation was not affected in differentiated neuronal cells as shown in Fig. 4-17. In this case also, close to 100% of
cells on 5 and 10 µm lanes were orientated less than 20 degrees of deviation from the reference line. Cells on 20 µm showed consistent behavior in terms of nuclear orientation when compared with undifferentiated cells. Fluctuations were observed among 30 and 40 µm ECM lanes as they provide ample space for multiple cells to attach in one spot.

![Figure 4-18: Neurite extension as a function of ECM lane width.](image)

Differentiation of neuronal cells using retinoic acid for 7 days significantly increased neurite extensions. These extensions were more prevalent in patterned cells compared to non-patterned cells. Differentiated non-patterned cells produced multiple neurites, while patterned differentiated cells had more organized neurite extension to connect with neighboring cells in the same lane. Neurite extension was significantly
increased in patterned cells in both conditions, i.e. differentiated cells and undifferentiated cells, when patterned in 5 and 10 µm. Fig. 4-18 shows that length of neurite extension in undifferentiated, patterned cells on 5 and 10 µm is significantly larger (p<0.001) than undifferentiated non-patterned cells. Similarly, same holds true for differentiated patterned cells in 5 and 10 µm ECM lanes with p value less than 0.001. However, there was no statistical significant difference between length of neurites on 5 and 10 µm ECM lanes in both cases. But, neurite length of differentiated cells on 5 and 10 µm were statistically larger (p< 0.01 and p< 0.001, respectively) than undifferentiated cells on the same width islands.

Discussions

Neuronal cell patterning and their confinement in defined geometry have been reported in previous studies (Branch et al., 1998; Branch et al., 2000; Branch et al., 2001; Corey et al., 1991; Frimat et al., 2010; Klein et al., 1999; Kleinfeld et al., 1988; Matsuzawa, Potember, Stenger, & Krauthamer, 1993; Morin et al., 2006; Nam, Branch, & Wheeler, 2006; Romanova, Fosser, Rubakhin, Nuzzo, & Sweedler, 2004; Scholl et al., 2000; Stenger et al., 1998; Suzuki et al., 2005; Thompson & Buettner, 2001; Wheeler et al., 1999; Yang, Co, & Ho, 2005a). Most of these studies were conducted to develop techniques for spatial organization of neuronal cells or for the sake of controlled neurite growth along a defined path. Organization of cells in different geometry alters stiffness and stress level of cells affecting their morphology and mechanics (Tee, Fu, Chen, & Janmey, 2011). Cellular morphology in turn controls various cellular functions through organization of cellular organelles. Therefore, understanding the influence of substrate surfaces and their geometry on cellular morphology would establish a better way of organizing cells for effective neuronal tissue regeneration or biomedical applications in
general. In this study, we have demonstrated that neuronal cell morphology and behavior can be altered by using different geometry of substrates that cells attached to.

Efficacy of PLL-g-PEG to confine different types of cells to different geometry has been reported in previous studies (Akiyama, Ito, Kawabe, & Kamihira, 2010; Branch et al., 2000; Branch et al., 2000; Branch et al., 2001; Branch et al., 2001; Csucs, Michel, Lussi, Textor, & Danuser, 2003; M. Zhang et al., 1998) (58,59,66,95,96). In this study, we have successfully immobilized PEG on TCP surfaces to confine SH-SY5Y on confined geometry of collagen-I lanes. PEG prevented cells from attaching or migrating to the areas where protein was not available. Attachment of secreted ECM proteins from cells or protein molecules from supplement media is also very limited for longer period of culture. Interestingly, our results suggest that confining of SH-SY5Y cells in smaller geometry such as 5 µm can only be achieved with application of PEG or similar protein and cell resisting chemicals. These cells tend to attach to the area beyond smaller ECM lanes due to very limited available space to attach. Moreover, cells in these smaller ECM lanes without backfilling molecules can attach randomly and decrease ordered organizations of cells and their nuclei. On the other hand, an application of PEG like molecules restricts cells to attach and grows along the main axis of protein patterns. Cells attached to smaller ECM islands (5 and 10 µm) did not grow as big as cells attached to bigger islands (20, 30 and 40 µm) or the controlled unpatterned cells in the same culturing condition. Cells require a critical area to properly attach and grow (Yan, Sun, & Ding, 2011) on the substrates. In our study, widths of ECM islands have greater influence in cell and nuclear spreading over their lengths, in accordance with previous studies (Yang, Co, & Ho, 2005a).

As mentioned earlier, due to critical area of attachment, number of attached cells is proportional to width of ECM lanes. Multiple cells randomly attached side to side in wider lanes. Upon their growth, some of the cells overlapped, and grow compactly covering entire width and length of ECM lanes making it complicated to measure number and length of neurite extensions. Application of neuronal biochemical factors enhanced multiple growths of neurites in SH-SY5Y cells. Patterned cells, especially in 5 and 10 µm
ECM lanes, showed significant increase in neurite length along the main axis of pattern. Also, total cell spreading areas of differentiated SH-SY5Y cells in these islands were statically equivalent to non-patterned, and or cells in bigger islands, when these excessively long neurite extensions were incorporated in total cellular spreading area. Despite of higher cell spreading area, nuclear areas were statistically compromised in smaller islands. This effect may lead to change in gene expression and protein synthesis as nuclear shape determines DNA organization (Ingber, 1999; Maniotis, Chen, & Ingber, 1997).

For both cases of differentiated and undifferentiated neuroblastoma cells, 5 and 10 µm ECM lanes produced longer and more organized growth of neurites. Differentiated SH-SY5Y cells on 5 µm statically had longest neurite extension when compared with control and other sample groups. In our study, 5 µm ECM lanes produced longest neurites length of 214.23 µm. Moreover, higher nodal compliance, cellular; and nuclear orientation and organization were achieved. Organization of neuronal cells in smaller cellular adhesive cues may provide an effective alternative for specific neuronal regeneration applications. However, due to compromised nuclear area in smaller geometry, further studies should be conducted to assess their effect in neuronal gene expression and protein synthesis.

Smaller geometries restrict cellular and nuclear spreading. In order to evaluate minimum suitable dimension of ECM islands for healthy growth and migration of neuronal cells, we selected bigger ECM lanes having width of 20, 30 and 40 µm. Cells and nuclear spreading areas in these patterns were significantly equal, but the nuclear organization found to be significantly ordered in 20 µm ECM lanes. These results suggest that growing cells in smaller pattern affect both nuclear and cellular spreading, and higher lanes produced randomization of nuclear organization.

In summary, cellular behaviors are affected by size of ECM lane widths. Incorporation of neuronal cells on smaller lanes may produce effective regeneration of neuronal cells in terms of neurite growth and organization of their nuclei. Similarly, In
terms of cellular and nuclear spreading, a minimum of 20 µm lanes is required for the healthy growth of cells.
Chapter 5: Conclusion

Through these studies, we have demonstrated a versatile method of cellular patterning for biomedical applications. Efficacies of this optimized microcontact patterning techniques have been demonstrated via reproducibility of different synthetic ECM protein patterns on 3 different biosurfaces, and confining three different cell lines on a wide range of microgeometry of above mentioned ECM protein patterns.

Using optimized techniques of photolithography and microcontact printing, in the second part of the study, we successfully confined SH-SY5Y human neuroblastoma cells in various geometry to control their morphology and cellular behaviors. Our results concluded that SH-SY5Y cells can be patterned to as low as 5 µm wide geometry to control their connectivity with other cells as well as their orientation. Cells conform to the geometry that they are constrained to grow but spreading area of cells and their nuclei is controlled by the width of the geometry of substrate surfaces. The 20 µm parallel lane was found to be optimal dimension for neuronal cell in terms of their growth. Highly ordered organization of cells and their connectivity can be obtained by confining cells on smaller width geometry, while an increase in their dimension influences randomization and disordered connectivity of cells. Similarly, for neuronal cells, longer neurite growth can be achieved by incorporating them on narrow lanes such as 5 and 10 µm widths, which might be useful for neuron regeneration or repair applications.

Our studies have shown PLL-g-PEG as an excellent cell and protein repellent molecule to organize and maintain spatial localization of cells for longer period of time. Differentiated neuronal cells on smaller islands may spread their area to the maximum but nuclear spreading is extremely constrained. Since nucleus is the hub for most of the cellular functions, they may also be affected with constrained geometry applied for it to spread.
Future direction

Our results show that behavior and morphology of SH-SY5Y human neuroblastoma cells can be modulated through different geometry and synthetic extracellular matrix proteins. Assessing of functions of these cells with altered morphologies and behavior will lead to proper nerve regeneration in-vitro.

Studies have reported cell seeding density also affects cellular morphology and behavior on patterned surfaces. Similarly, biochemical features of ECM proteins have also been reported as a cause to altered cell morphologies and functions. Human neuroblastoma cells occur in three different sub types, S-type (e.g. SH-EP), N- type (e.g. SH-SY5Y) and I-type (e.g. IMR-32). These cells may behave independently in terms of attachment, migration and proliferation on different substrates.

Therefore, further investigation including all of these 3 different neuronal cell types, with varying cell seeding densities on ECM patterns of different geometry will lead us toward better understanding and control of neuron cell morphology. Quantitative analysis of various human neuronal marker genes (e.g. MAP2, ChAT, NFH, GAD 67, NFM, NSE, PAP, Peripherin, Beta-Tubulin 3, TAU) expressed due to these constrained microenvironments will provide breakthrough progress toward neuronal regenerative medicines and biomedical applications.
References


Tsai, M. T., Lin, D. J., Huang, S., Lin, H. T., & Chang, W. H. (2011). Osteogenic differentiation is synergistically influenced by osteoinductive treatment and direct cell-cell contact between murine osteoblasts and mesenchymal stem cells. *International Orthopaedics,*


