Engineering Substrates for the Study of Cell Mechanical Interactions

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ABSTRACT

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This thesis describes the effect of geometries with controlled radius of curvature on cellular behavior, a novel approach to measure and map protrusive forces in cells and the application of fabrication techniques like wet etching and microcontact printing to answer fundamental biological problems.

In order to isolate the effects of curvature from other factors, we have developed a technique to create features with nominally identical dimensions but varying radius of curvature. Using these substrates, we analyzed the effect of curvature on cell morphology. Cell area and aspect ratio were examined on various substrates, and immunostaining of focal adhesions, stress fibers and microtubules were used to show the effect of curvature on these cytoskeleton components. We show that feature curvature has an effect on both cell morphology and cytoskeleton organization. Using this technique, it may be possible to engineer precise geometries that can lead to better design of scaffolds and biomaterials for tissue engineering.

The motivation behind measuring cellular forces was two fold, first to measure the protrusive forces locally and with spatial resolution and second to measure them at the same time as traction forces. This is important because cell motility is a result of forces
generated within a cell and various biological processes like cancer metastasis, wound healing and immune response are a result of cell motility. Thus, measuring these forces precisely and simultaneously will help us to design and develop devices that can have application in cancer diagnostics and wound healing therapies. The magnitude of protrusive force measured was 1.0 nN and traction force was computed to be 2.7 nN. Furthermore we also estimated the number of actin filaments per micron square which agree with previously reported values thus confirming the accuracy of this method. The approach presented here is the first study to simultaneously measure the protrusive and traction forces in cells.

In chapter 4, I describe in detail the fabrication process for making high aspect ratio grooves and ridges by wet etching Silicon using boiling potassium hydroxide. The etched substrates were used as imprint masters and were faithfully replicated and molded in a silicone elastomer. Next the substrates were plasma fluorinated and used to form elastomer stamps for microcontact printing and other applications requiring easy mold release.

In chapter 5, I have used microcontact printing to fabricate substrates to help understand plasma membrane dynamics. The role of plasma membrane (PM) area as a critical factor during cell motility is poorly understood, mainly due to an inability to precisely follow PM area dynamics. To address this fundamental question, we developed static and dynamic assays to follow PM area changes during fibroblast spreading.
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“yogarato va bhogarato va
sangarato va sangavihinah
yasya brahmani ramate chittam
nandati nandati nandatyeva”
Chapter 1

Background and motivation

1.1 MECHANICAL FACTORS GOVERNING CELLULAR RESPONSE AND RELEVANCE TO PHYSIOLOGICAL SYSTEMS

Mechanobiology is the study of the role of mechanical factors like geometry, force and rigidity on cells. The two main aspects of mechanobiology are: first, to elucidate the mechanosensing, transduction and response mechanisms through which cells modulate their functions and second, the measurement and characterization of cellular mechanical properties. Geometry and force are the two properties sensed by the cells at the nano to micrometer scale. Local sensing of these is transduced into biochemical signals that result in cell responses [1]. Cells face diverse biomechanical environments and respond to externally applied or internally generated mechanical stress, such as changes in plasma membrane tension by the topography and rigidity of ECM, shear stress, hydrostatic pressure, and compression in a human body [2]. Extracellular matrix (ECM) proteins exhibit abundant nanometer-scale structures that are hypothesized to contribute to cell – matrix signaling [3]. Tissue and organ formation are a result of complex cell – substrate interactions that involve various processes like adhesion, migration, proliferation and differentiation. Fig. 1.1 shows the various biomechanical environments of cells and tissue in a human body. Additionally, various other processes like morphogenesis, wound healing, cancer metastasis and immune response are a result of cell movement and motility. For example for wound healing to occur, white blood cells and macrophages move to the wound site to kill the
microorganisms that cause infection, and fibroblasts (connective tissue cells) move there to remodel damaged structures [4]. A lot of work has been done in understanding the effects of biochemical factors on cellular response, however the effect of geometry and force that the cell is exposed to has been less understood. Recent advances in nanofabrication have equipped researchers with various tools to better engineer and understand cellular mechanotransduction and mechanosensing.
Figure 1.1. The biomechanical environment of cells and tissues in a human body. Cells experience various mechanical stimuli in a human body. (a,b) In blood vessels, cells are continuously subjected to shear and hydrostatic stress from blood flow. (c) Tissues in lung and heart are stretched cyclically during breathing and heart beating. (d) Cell motility and tissue organization are affected by mechanical and structural properties of extracellular matrix (ECM) components. For instance, gradients and structural organization of matrix proteins appear to provide guidance cues to myocardial alignment during cardiovascular development (c) as well as directional organization of cells in connective tissue. (e) Cancer cells spread from a primary tumor site and invade other organs. (f) Another example is a skin wound [2].

1.2 STEPS IN MECHANOSENSING

Fig. 1.2 shows the steps in mechanosensing over time that involves feedback loop between inside and outside of the cell. First the cells sense the mechanical features of their environment, which causes rapid motility and signaling responses; due to this the extracellular matrix is modified and creates new signals. Intracellular signals alter protein expression of the cell. Thus, cells are continually encountering new ligands and the structural and signaling functions of ECM-integrin-cytoskeleton molecular complexes are constantly modified depending on the magnitude of the forces/geometry they transduce.
Figure 1.2. A schematic of the feedback loops that connects the intracellular and extracellular cellular events. Cells sense the mechanical features of the environment and modify the ECM and the protein content. Intracellular signals will also alter the protein expression and therefore cell function [1].

1.3 COMPONENTS INVOLVED MECHANOTRANSDUCTION

1.3.1 Cytoskeleton

The cytoskeleton is a meshwork of different sets of polymeric proteins that provide structural support to the cells and stabilize cell shape. As such, it is the main determinant of cell viscoelasticity, even though it also plays a crucial role in functions such as intracellular trafficking, cell division, or crawling (in adherent cells). Its main components are actin microfilaments, microtubules, and intermediate filaments, along with the associated proteins that bind to them.
Figure 1.3. Structure and electron microscopy images of the different cytoskeletal proteins. 
(a) Actin microfilaments. (b) Microtubules. [4]

1.3.2 Actin microfilaments

Microfilaments are linear polymers of the monomeric form of actin, known as globular actin (G-actin). Microfilaments have a diameter of ~8 nm, a length that can reach up to 30-100 μm, and have a polar configuration (Fig. 1.3a) Certainly, microfilaments have a pointed end, with low polymerization rates, and a barbed end, with higher polymerization rates and growth. Actin is the most abundant protein in most eukaryotic cells, accounting for about 5 - 10% of the total protein content. Actin cytoskeleton is disrupted using different actin-binding drugs (such as cytochalasins or latrunculin).
1.3.3 Microtubules

Microtubules are formed by the polymerization of tubulin dimers, which form hollow cylinders of ~25 nm diameter that can be more than 100 µm long. These cylinders have a distinct polarity, with a plus end that recruits additional dimers faster than the minus end (Fig. 1.3b). The amount of microtubules present in cells is much smaller than that of microfilaments, and their respective organizations are very different. Microtubules radiate individually from a nucleating center called the centrosome. Additionally, individual microtubules are very unstable structures, which randomly switch from net polymerization and growth to net depolymerization and shrinkage in a process termed dynamic instability. Microtubules can be disrupted using pharmacological inhibitors like nocodazole or colchicine.

1.3.4 Intermediate filaments

The mechanical role of intermediate filaments remains unclear, although disruption of vimentin filaments with acrylamide or calyculin A was reported to reduce stiffness in non adhesive T lymphocytes [5] and in adhesive fibroblasts and endothelial cells [6]. For the study presented in this thesis we have not focused on intermediate filaments.

1.3.5 Integrins and focal adhesions

Cells adhere to and interact with their substrate through surface receptors, most notably integrins (Fig. 1.4). Integrins are a family of transmembrane heterodimeric glycoproteins that physically link the extra cellular matrix (ECM), the cell surface and the intracellular cytoskeleton [7, 8]. Intergrins consists of two non-covalentely associated subunits - α and β and specific combination of subunits interact with specific amino acid sequences, ligands and complex proteins. Binding of integrins to the ECM results in clustering and recruitment
of scaffolding proteins inside the cell that help the integrins to physically connect to the actin cytoskeleton at the sites of focal adhesion [9].

![Diagram of Integrin Structure](image)

Figure 1.4. Subunit structure of an active integrin molecule, linking extra cellular matrix to the actin cytoskeleton [4].

The ability of integrins to convert extracellular responses is mediated by the proteins localized at the interface between the receptor and the cytoskeleton, called the focal adhesion complex. Focal adhesion complex are large macromolecular assemblies, through which cells communicate with the ECM, both in terms of cell-ECM attachment and signal transduction. These assemblies are enormously complex, and contain over 100 different proteins [10].
1.4 The scope of this work

The major part of the work presented in this thesis studies the effect of geometry on cellular mechanotransduction and measurement of protrusive forces in cells. To differentiate between the two studies, I will first give an overview of geometry sensing in cells and then I will move on to describe the forces in cells, and techniques employed to measure the forces. Chapters 2 and 3 will describe the experiments in detail.

1.5 CELLULAR RESPONSE ON NANO/MICRO TOPOGRAPHIES (GEOMETRY SENSING)

Surface topography has critical effects on the formation of tissues, for instance the topology of a scaffold in an implant will govern whether the cells will attach and proliferate on the substrate. An increase in surface area may provide greater potential for tissue integration (mechanical interlocking) [11]. Cells sense changes in substrate topology and respond by modifying their behavior this phenomenon is called contact guidance. Thus, through the appropriate engineering of physical aspects of the environment we can regulate the cell function. Nanofabrication can help us create various in vivo three – dimensional ECM environments with structural and mechanical similarity. Experimental studies with various nanotopographic features such as grooves, ridges, pillars, wells has shown that topography has a strong influence on cell morphology, proliferation, migration and gene expression (Fig. 1.5). Table 1 and 2 lists the behavior of different cells types on different topographies. However, the role of controlled substrate curvature in contact guidance remains elusive.
Figure 1.5. Example of various substrates used for geometry sensing. (a) Schematic depictions and (b) SEM images of representative various topographies. Three basic nanotopography geometries include nanograting (45° tilt, scale bar 5 mm), nanopost array (15° tilt, scale bar 5 mm), and nanopit array (scale bar 1 mm) [3].
<table>
<thead>
<tr>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Nanograting size</td>
</tr>
<tr>
<td>600 nm</td>
</tr>
<tr>
<td>PDMS</td>
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<tr>
<td>+ +</td>
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<td>--</td>
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<tr>
<td>+ +</td>
</tr>
<tr>
<td>Organized into cellular superstructures</td>
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<table>
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<th>Stem cells</th>
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<td>750 nm</td>
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<td>+</td>
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<td>--</td>
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<tr>
<td>Cytoskeleton disrupting agents</td>
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<table>
<thead>
<tr>
<th>Hematopoietic stem cells</th>
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</thead>
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<tr>
<td>600 nm</td>
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<tr>
<td>PDMS</td>
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<tr>
<td>+ +</td>
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<td>--</td>
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<td>--</td>
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<tr>
<td>Cytoplasmic disruption</td>
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</table>

<table>
<thead>
<tr>
<th>Mesenchymal stem cells</th>
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<tbody>
<tr>
<td>350 nm</td>
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<tr>
<td>PDMS</td>
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<td>+ +</td>
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<tr>
<td>Differentiation into neural lineage</td>
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<table>
<thead>
<tr>
<th>C6 glioma</th>
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<tbody>
<tr>
<td>266 nm</td>
</tr>
<tr>
<td>PS</td>
</tr>
<tr>
<td>++</td>
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<td>--</td>
</tr>
<tr>
<td>--</td>
</tr>
<tr>
<td>Neuronal lineage</td>
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<table>
<thead>
<tr>
<th>hEKC (HEK-293)</th>
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<tbody>
<tr>
<td>200–430 nm</td>
</tr>
<tr>
<td>PS</td>
</tr>
<tr>
<td>++</td>
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<td>--</td>
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<tr>
<td>Polarized MTOC</td>
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<table>
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<tr>
<th>Smooth muscle cells</th>
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<tbody>
<tr>
<td>350 nm</td>
</tr>
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<td>PDMS, PMMA</td>
</tr>
<tr>
<td>+ +</td>
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<td>--</td>
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</tr>
<tr>
<td>Increase in fibronectin mRNA, incorporation</td>
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</table>

<table>
<thead>
<tr>
<th>Fibroblasts</th>
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<tbody>
<tr>
<td>3–5 µm</td>
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<tr>
<td>Ti</td>
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<tr>
<td>+ +</td>
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<tr>
<td>Increase in fibronectin mRNA</td>
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<thead>
<tr>
<th>Corneal ECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>70–2100 nm</td>
</tr>
<tr>
<td>SI</td>
</tr>
<tr>
<td>+ +</td>
</tr>
<tr>
<td>--</td>
</tr>
<tr>
<td>--</td>
</tr>
<tr>
<td>Biased lamellipodia</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Corneal ECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–20 µm</td>
</tr>
<tr>
<td>PS</td>
</tr>
<tr>
<td>++</td>
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<tr>
<td>--</td>
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<tr>
<td>--</td>
</tr>
<tr>
<td>Study of coupled topography, electric field, and soluble factors</td>
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</table>

<table>
<thead>
<tr>
<th>Corneal ECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4 µm</td>
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<tr>
<td>Quartz</td>
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<tr>
<td>++</td>
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<td>--</td>
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<tr>
<td>--</td>
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<tr>
<td>Cooperative neurite extension</td>
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</tbody>
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<table>
<thead>
<tr>
<th>PC12</th>
</tr>
</thead>
<tbody>
<tr>
<td>70–1900 nm</td>
</tr>
<tr>
<td>SI</td>
</tr>
<tr>
<td>++</td>
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<tr>
<td>--</td>
</tr>
<tr>
<td>--</td>
</tr>
<tr>
<td>Cooperative neurite extension</td>
</tr>
</tbody>
</table>

[a] No entry: data not available; ++: increase under all conditions; +: increase under most conditions; 0: no detectable change; --: decrease under most conditions; -: decrease under all conditions. [b] h, b, and r indicate human, bovine, and rat cells, respectively. [c] PDMS = poly(dimethyl siloxane), PS = polystyrene, PCS = poly(glycerol sebacate), PMMA = poly(methyl methacrylate). [d] MTOC = microtubule organization center

Table 1.1. Response to nanogratings [3].
Table 1.2. Response to nanopost and nanopits [3].

1.6 CURVATURE SENSING IN CELLS

Not many studies have studied the effect of substrate curvature on cellular mechanotransduction. One theoretical study by Herrera et.al [12] showed that substrate curvature has two implications: (i) it forces fibers to work in a curved (bent) position and (ii) it eventually creates a pre-deformation state in the cytoskeleton. Interestingly, their model shows higher contractile force inhibition as curvature increases when implemented over different substrate morphologies (Fig. 1.6 and 1.7).
Figure 1.6. Mechanical system of a cell filament bundle oriented at a certain spatial direction featured by q. The main elements that provide mechanical resistance to the cell cytoskeleton (CSK) are classified into passive (Epas) and active elements; which in turn is composed by the contractile actomyosin apparatus AM (see Fig. 2d) and the actin elasticity Eact [12].
Figure 1.7. Model of the contractile apparatus of the cell anchored to a curved substrate/ECM. Cell feels the local curvature at the CSK level regulating its contractile force (a). At a higher observation level (b), actin filaments are distributed along different spatial directions. (c) Maximum force exerted by the myosin II contractile machinery (referred to an undeformed state) over the direction of the actin filaments considering the local substrate curvature. The profile of the contractile pressure $p_c$ (d) is expressed as a function of the deformation of the actin filaments, and the local substrate curvature through $p_{max}$ ($k_1 < k_2$) [12].

Another study (Fig. 1.8) showed that when rat melanoma cells are exposed to microcontact printed geometries with local curvature, there is strong localization of actin based cytoskeletal structures on the adhesive islands [13].
Figure 1.8. (1) Geometric cues presented by adhesive islands. (2) Local curvature influences the distribution of actin-based structures in adherent cells. Cells were allowed to spread onto adhesive islands of different shapes and were fixed and stained for actin (B, F, J) and cortactin (C, G, K). Representative images of single cells are shown together with a heat map for cortactin generated from a population of cells (D, H, L). Scale bar - 20 μm [13].

Rumpler and co workers [14] examined the effect of curvature on three dimensional tissue growth and found that tissue growth always started in the corners of the channels (large local negative curvature), while cells on the channel faces (zero curvature) produced tissue only when their neighborhood became curved from tissue growing outward from the corners (Fig. 1.9).
Figure 1.9. (a) New tissue formed in three-dimensional matrix channels. Actin stress fibers are stained with phalloidin-FITC and visualized under a confocal laser scanning microscope. Here, the tissue formation is shown (i–iii) after 21 days and (iv) after 30 days of cell culture in the channels of a (i) triangular, (ii) square, (iii) hexagonal and (iv) round shape introduced into a HA plate in vitro. (b) Numerical simulation of tissue formation within channels of various shapes: (i) triangular, (ii) square, (iii) hexagonal and (iv) round. The lines (early time point 1, ongoing times 2 and 3) mark the simulated development of tissue formation due to ongoing culture time, which corresponds closely to the observed development of new tissue formation in vitro [14].

1.7 FORCES IN CELLS

Mechanical forces inside and outside the cells help in regulating various physiological and pathological processes. The forces generated inside cells are of two types – protrusive and traction. Protrusive forces are a result of actin polymerization at the leading edge of the lamellipodia. On the other hand traction forces are the forces generated by the cell against the substrate in order to move forward, given its adhesion to the substrate [15]. Both these
forces help in cell movement as a response to external physical or chemical cue. Fig. 1.10 shows the stages of cell movement.

Figure 1.10. Schematic of three stages of cell movement [4].

Briefly, once the direction of motion is determined the cell extends protrusion n that direction. It then adheres its leading edge to the surface on which it is moving and de adheres the cell body and rear. Finally, it pulls the whole cell body forward by contractile forces generated at the cell body and rear of the cell.
1.7.1 Methods to measure traction forces

Various methods have been used to detect and quantify the traction forces generated by single cells (Fig. 1.11).

![Images of various tools for measuring cellular forces](image)

Figure 1.11. Micro- and nanoscale tools for the measurement of cellular forces. (A) Silicon rubber membrane wrinkles due to the traction forces from a fibroblast. Bar: 50 μm. (B) Traction force microscopy reports the traction forces from a migrating fibroblast (arrow indicates direction) by measuring the displacement of fluorescent microbeads (0.2 μm) embedded in a gel substrate. (C) Regular array of micropatterned fluorescent dots distorts underneath a contracting fibroblast. (D) Bending of horizontal microcantilever locally reports the traction force during fibroblast migration. (E) Array of vertical elastomeric microcantilevers bend and report the localized contractile forces of smooth muscle cell. Scale bar - 10 μm [16-20].

Table 3 summarize the various techniques used and the magnitude of forces obtained.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Traction Force (Magnitude)</th>
<th>Stall Force (Magnitude)</th>
<th>Measurement Location</th>
<th>Measurement Technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>91.5 pN/μm²</td>
<td>&gt;8.5*10⁴ pN/μm²</td>
<td>deformation of polyacrylamide substrate</td>
<td>[84]</td>
<td></td>
</tr>
<tr>
<td>(normal)</td>
<td>~3000 pN/μm²</td>
<td>(average traction)</td>
<td>deformation of polyacrylamide substrate</td>
<td>[58]</td>
<td></td>
</tr>
<tr>
<td>(cancerous)</td>
<td>~997 pN/μm²</td>
<td>(average traction)</td>
<td>deformation of polyacrylamide substrate</td>
<td>[85]</td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>~6000 pN/μm²</td>
<td>front of cell</td>
<td>deformation of polyacrylamide substrate</td>
<td>[85]</td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>8700/-430 pN/μm²</td>
<td>front of cell</td>
<td>micromachined device</td>
<td>[86]</td>
<td></td>
</tr>
<tr>
<td>(average force exerted by cell on the substrate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratocyte</td>
<td>~200000 pN</td>
<td>displacement of beads in silicone substrate</td>
<td>[57]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(max. traction force)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratocyte</td>
<td>~10000 pN</td>
<td>deformation of silicone substrate</td>
<td>[88]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(propulsive traction force of stuck cell)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratocyte</td>
<td>~45000 pN</td>
<td>whole cell</td>
<td>deformation of silicone substrate</td>
<td>[8]</td>
<td></td>
</tr>
<tr>
<td>Keratocyte</td>
<td>~1180 pN 40000 pN</td>
<td>lamellipodium</td>
<td>AFM tip in path of moving cell</td>
<td>[82]</td>
<td></td>
</tr>
<tr>
<td>Keratocyte</td>
<td>400-2400 pN 34000-85000 pN</td>
<td>whole cell</td>
<td>AFM tip in path of moving cell</td>
<td>[7]</td>
<td></td>
</tr>
<tr>
<td>Keratocyte</td>
<td>0.5-13 pN</td>
<td>tip of lamellipodium</td>
<td>water flow from pipette to locally stall cell</td>
<td>[81]</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3. Magnitude of various forces measured in cells [15].

Although a lot of studies have shown the measurement of traction forces, not many studies have measured the protrusive forces exerted by the cell.
1.7.2 Measurement of protrusive forces

Protrusive forces are developed by actin polymerization at the leading edge of the cell and help in lamellipodial movement. Not many studies have measured this force, the reason being the difficulty in measurement. As these forces are produced in lamellipodia which is a broad sheet like structure 100 – 200 nm thick [21], it is difficult to measure the forces generated by this sheet. Two studies have reported the measurement of protrusive force in fish keratocytes.

In one study by Prass et.al [22] an atomic force microscopy cantilever was placed in the path of a migrating keratocyte and the deflection of the cantilever provides a direct measurement of protrusive forces exerted by the lamellipodial leading edge (Fig. 1.12).
Figure 1.12. (a) Schematic of the measurement process. (b) Micrograph of the base of a cantilever, indicating the region of contact with the lamellipod (c) Series of images of direct force measurement at t = 0, 30, 60, 233, and 260 s, from left to right. Small white line, initial position of the cantilever; large white line, current dorsal part of the cell; dashed white line, large white line from the previous image. Scale Bar - 5 μm [22].

Bohnet and co-workers [23] arrested the leading edge of a moving fish keratocyte with a hydrodynamic load generated by a fluid flow from a micropipette. The flow arrests protrusion locally as the cell approaches the pipette, causing an arc-shaped indentation and upward folding of the leading edge. Modeling of the fluid flow gives a value for the arresting force of few piconewtons per micrometer (Fig. 1.13).
Figure 1.13. Reversible arrest of the leading edge by a hydrodynamic load.

(A) (0–30) As the cell approaches the pipette tip, the protrusion becomes locally arrested by the flow, resulting in the arcshaped indentation of the leading edge. (37–104) The leading edge recovers its initial shape when the pipette is removed. (B) Traces of the position of the leading edge and the front boundary of the cell body. Whereas the leading edge of the cell is arrested by the flow and then recovers after the pipette removal, the cell body translocation unaffected. (c) Flow parallel to the leading edge neither stops the protrusion nor affects cell motility in general. L stands for the lamellipodium and B for the cell body.

Scale Bar - 10 mm; time in seconds [23].
1.8 GOALS OF THIS STUDY

In this work, we explicitly examine the role of curvature in geometry sensing. In order to isolate the effects of curvature from other factors, we have developed a technique to create features with nominally identical dimensions but varying radius of curvature. Using the above substrates, we analyzed the effect of curvature on cell morphology. Cell area and aspect ratio were examined on various substrates, and immunostaining of focal adhesions, stress fibers and microtubules were used to show the effect of curvature on these cytoskeleton components. We show that feature curvature has an effect on both cell morphology and cytoskeleton organization. Using this technique, it may be possible to engineer precise geometries that can lead to better design of scaffolds and biomaterials for tissue engineering.

Chapter 2 describes the measurement of protrusive force using a silicone elastomer device. The motivation behind this study was two fold: first, to measure the protrusive forces locally and with spatial resolution and second, to measure them at the same time as traction forces. The approach presented here is a simple and novel way to measure the protrusive and traction forces in cells. The advantages of this device are:

1. Multiple force sensors for analysis on a single device.
2. Unperturbed measurement of cellular forces thus giving more precise measurements.

Chapter 4 describes the fabrication of grooves and ridges using silicon <110>. Here we used boiling potassium hydroxide (KOH) solution to etch silicon. Grooves and ridges with different heights were fabricated and the substrates had vertical sidewalls and smooth surfaces and used for a study of making an anti adhesion coating by an exposure to fluorocarbon – based plasma.
Chapter 5 describes the fabrication of microcontact printed circles with varying diameter that were used to better understand the role of plasma membrane (PM) area during cell spreading.
Chapter 1. Background and Motivation

References


Chapter 2

2.1 INTRODUCTION

The mechanical properties of a cell’s environment play a large role in determining cell behavior and phenotype: the interplay between mechanical and biochemical signals influences responses that regulate cell growth, differentiation, shape change and cell death [1]. Within the field of mechanosensing, one area of particular interest is cellular interactions with surface topography: numerous studies have shown that cells react to underlying topographical features like grooves and ridges by modifying their cytoskeleton and aligning to the topography. This phenomenon is referred to as contact guidance [2].

A great deal of recent work has explored contact guidance in a variety of systems. Epithelial cells, keratocytes, neuronal cells and smooth muscle cells [3-6] all exhibit contact guidance on grooves and ridges by polarizing along the features. Fibroblasts respond both to feature size and feature density [7]. Furthermore, it has been shown that human corneal epithelial cells can elongate and align to ridges with widths as small as 70 nm [4]. Loesberg et al [8] showed that fibroblasts respond to grooved patterns with a height and width of 35 nm and 100 nm, respectively. Fibroblast and neurons plated on Ni nanowires for 24 and 72 h respectively display contact guidance [9]. Moreover, substrate topography has also been shown to influence cell differentiation. Human mesenchymal stem cells (hMSC) on polymethyl methacrylate (PMMA) nanopit arrays produced bone specific extra cellular matrix (ECM) proteins, despite the absence of osteogenetic supplements [10].

One of the mechanical factors that has received little attention to date is the curvature of features in the external environment: only a few studies have examined the
effect of curvature on cell mechanotransduction. In one study, it was reported that the amount of tissue deposited is proportional to the local curvature [11], a finding that could be important in the field of tissue engineering and designing artificial implants. Endothelial cells on curved surfaces respond to flow rapidly, with marked changes in filamentous actin central stress fiber formation [12]. When rat melanoma cells are exposed to microcontact printed geometries with local curvature, there is strong localization of actin based cytoskeletal structures on the adhesive islands [13]. Herrera et al [14] used a multiscale modeling approach and reported that increased curvature leads to a higher inhibition of contractile force. In the previous experimental work, the structures used had radii of curvature on the micron scale and above. However, because cells in the body spread on ECM fibrils with diameters between 260 and 410 nm [15], it is important to examine the role of feature curvature in this size range.

In this work, we explicitly examine the role of curvature in contact guidance. In order to isolate the effects of curvature from other factors, we have developed a technique to create features with nominally identical dimensions but varying radius of curvature. The technique used to fabricate these features is described in the fabrication section. Briefly, photolithography and plasma etching were used to generate an array of sharp lines with width \( w' \), height \( h \), and pitch \( p \) on a fused silica substrate. Next, a layer of silicon dioxide of thickness \( r \) was conformally deposited onto the substrate to give rounded features with width \( w = w' + r \), radius of curvature \( r \), and height \( h \). By using starting patterns with appropriate \( w' \), a series of substrates with identical \( w \) and \( h \), and \( p \) could be generated, with only \( r \) varying from substrate to substrate. The details of the process are given below in the fabrication section.

We note that choosing the proper material for the substrate is crucial since the low optical contrast of cells requires a transparent substrate for transmission microscopy.
Chapter 2. Role of Curvature in Contact Guidance

Because glass does not etch uniformly, fused silica (170µm thick) wafers were used [16]. In addition it also ensured that any effect we observe is only due to change in geometry and not due to rigidity changes, which could be the case for other commonly-used transparent materials such as silicone elastomers.

Using the above substrates, we analyzed the effect of curvature on cell morphology. Cell area and aspect ratio were examined on various substrates, and immunostaining of focal adhesions, stress fibers and microtubules were used to show the effect of curvature on these cytoskeleton components. We observe that feature curvature has a profound effect on both cell morphology and cytoskeleton organization. Specifically, increased radius of curvature decreases cell polarization. Using this technique, it may be possible to engineer precise geometries that can lead to better design of scaffolds and biomaterials for tissue engineering.

2.2 EXPERIMENTAL

2.2.1 Fabrication of substrates

The fabrication of the substrates was challenging, as smooth, clean and transparent substrates were required to study the cellular behavior. The obvious choice to start with was glass cover slips and different though unsuccessful methods were tried on glass substrates. Finally fused silica was chosen as a substrate material. Four different methods were tried to make the curved features, these were - wet etching of fused silica and silicon dioxide, resist reflow and spacer technique.
2.2.1.1 Sharp substrates

2.2.1.1.1 Single layer resist process on glass substrates

Fig. 2.1 shows the schematic of the fabrication process used. Glass cover slips (22x22 mm, 170 μm thick) were subjected to 45 min nanostrip clean, then rinsed with DI water for 5 min, followed by a rinse with acetone and isopropanol, and finally dried with a nitrogen gun. The substrates were spin - coated with 300 nm thick ebeam resist polymethylmethacrylate (PMMA), molecular weight 495K plus 6% anisole (Microchem) and baked on a hot plate at 180 °C for 60 s to drive residual solvents away from the ebeam resist film. Followed by a layer of Aquasave conducting polymer (Mitsubishi Rayon) to prevent charging during e beam lithography. The thickness of obtained film was measured by Null ellipsometer (Rudolf El III). An FEI XL 30 Sirion scanning electron microscope equipped with a Nabity NPGS pattern generator was used for the patterning, with the exposure conditions of 5 keV and a probe current of 10 μA. After the pattern was written the Aquasave was washed off with deionized water and the sample dried with N2. Resist was developed using a 1:3 solution of methyl isobutyl ketone (MIBK) to isopropanol for 1 min with ultrasonic agitation at 5 °C. The substrate was then rinsed in isopropanol and dried with N2. To remove any residual resist layer the sample was descum in oxygen plasma for 1 min. Next, a thin layer of chromium (Cr) was deposited using a Semicore electron beam evaporator. For this work, Cr film of 50 nm was used. Finally, the coated substrate was submerged in a 65 °C acetone bath for 45 min for metal lift-off, and then sonicated for 1 min. Following lift-off, samples were rinsed with acetone to remove stray chromium and dried with N2. The patterned masters were then reactive ion etched to a depth of 500 nm in a mixture of C4F8:O2 (18:3) for 25 minutes at a ICP power of 300 W, rf power of 100 W and pressure 8 mT using an Oxford ICP etch tool.
Figure 2.1. Schematic of fabrication of substrates in glass using single layer resist.

But the pattern obtained had two issues:

1. Some areas did not have a clean lift off and resist was still stuck on some areas (Fig. 2.2a,b). Primary reason for this is that the metal film is deposited on the sidewall of the resist, and adheres to the substrate even after the resist removal. When these samples were etched the areas of the pattern that still had resist did not etch and micromasked the substrates thus creating the defects (Fig. 2.2c,d).

2. The glass substrates post etching turned translucent, the reason for this being the deposition of polymer during the etching process. This happens because, first unclean lift off leaves a coating of resist and second, glass has impurities like B\textsubscript{2}O\textsubscript{3}, Na\textsubscript{2}O, Al\textsubscript{2}O\textsubscript{3}, Fe\textsubscript{2}O\textsubscript{3}, CaO, MgO, and Cl that interfere during plasma etching and lead to a polymer build up.
Figure 2.2. SEM images of exposed and etched samples. (a) Improper lift off leaves resist residue on sample. (b) Etched samples with resist in between the ridges. (c, d) Micromasking of samples leads to a rough surface.

2.2.1.1.2 Bi Layer process

To obtain a clean lift off, we developed a bilayer resist process. This process is useful when an undercut profile is required. The principle behind this is, at the same exposure dose, the development rate for low molecular weight resist is faster than that for high molecular weight resist, resulting in broadened profile at the lower section of the pattern. In this case the bilayer stack was a combination of top layer of PMMA 495 A6 and bottom layer of a copolymer of PMMA – Methylmethacrylate (MMA-17.5)- methacrylic acid (MAA) - Ethyl
lactate (EL-6)(Microchem). The schematic of the fabrication is shown in Fig. 2.3 and explained below.

![Fabrication Schematic](image)

**Figure 2.3.** Schematic of the bi layer process on glass.

Clean substrates (as in section 2.3.1.1.1) were spin coated with a 190 nm thick copolymer and baked on a hot plate at 150 °C for 3 min. Followed by spin coating a 300 nm thick layer of PMMA 495 A6 and baked on a hot plate at 150 °C for 3 min. Finally, conducting polymer Aquasave was spun on the substrates. The coated substrate was exposed to e-beam at 5 keV, a probe current of 10 µA and an exposure dose of 650 µc/cm². Post exposure the samples were subjected to the same process flow (as in section 2.3.1.1.1). Fig. 2.4 shows the undercut profile of resist, width of undercut increases with an increase in the exposure dose.
Figure 2.4. SEM of undercut resist profile with bottom layer – copolymer and top layer PMMA 495 A6. (a) Exposure dose 650 μc/cm². (b) Exposure dose 800 μc/cm². Increasing the exposure dose increases the undercut.

Clean lift off was obtained and samples were plasma etched. Although the surface was free of any impurities, the etching left the samples rough, which was verified using an AFM. Fig. 2.5 shows the etched sample, note that there is no micromasking in this case.

Figure 2.5. SEM of etched glass, no micromasking but the surface was rough.
2.2.1.2 Fused Silica substrate

To address the problem of polymer deposition and rough surface we changed the substrate material to fused silica (170 μm thick). We note that for this study choosing the proper material for the substrate is crucial since the low optical contrast of cells requires a transparent substrate for transmission microscopy. Fig. 2.6 shows the schematic of the fabrication process. RCA cleaned fused silica wafers were covered with a 170nm layer of XHRiC i-line anti-reflection coating (ARC) polymer (Brewer Sciecne) baked at 180°C for 60 s on a hot plate. SPR 700 resist was then spun on the substrates, and soft baked at 90°C for 60 s. The wafers were patterned by UV photolithography in a GCA Autostep 200 system, postexposure-baked at 115°C for 60 s, and manually developed for 60 s with AZ 300 MIF developer.

Figure 2.6. Schematic of the fabrication process for sharp substrates on fused silica. (a – e) Process flow. (f) SEM of exposed resist with vertical side walls. (g) SEM of etched fused silica. SEM images taken on a 45° stage.
Fig 2.6(f) shows the resist profile after exposure and development, it can be seen that the side wall is vertical and the exposed area is clean. To remove any leftover resist and ARC, a 75 s O₂ plasma descum was performed. With the patterned resist as a mask, the fused silica was etched by reactive ion etching (RIE) (Oxford PlasmaLab 80+), using O₂ (2 sccm) and CHF₃ (50 sccm) gases at 40 mTorr and RF power of 240 W. Etching times from 1.7 minutes to 34 minutes were used to fabricate substrates with heights from 50 nm – 1 μm. Fig 2.6(g), shows the etched sample etched to a height 1 μm. The samples obtained by this technique were clean i.e. with no polymer buildup and the roughness was less than 8 nm as verified by AFM. Additionally, for all the samples the height was verified using AFM as shown in Fig. 2.7.

Figure 2.7. AFM measurements for height characterization of samples. (a) Height of features - 200 nm. (b) Height of features – 400 nm.
2.2.1.2.1 Purpose of Anti Reflective Coating (ARC)

Use of ARC is critical in this method as ARC prevents light from reflecting against the substrate. Reflected light results in standing waves in the resist that spoil the sidewall profile of the resist. ARC absorbs the light entering the material and a correct thickness causes destructive interference of the reflected light (Fig. 2.8).

![Diagram of destructive interference](image)

Figure 2.8. (a) Schematic of destructive interference of light entering the samples coated with ARC. (b) Reflectivity curve for ARC [Brewer Science general ARC presentation].

2.2.1.3 Fabrication of curved substrates

Fabrication of curved substrates was more challenging than fabrication of sharp substrates. The primary difficulty being the precise control of width, height and pitch with the only
dimension changing - radius of curvature. For this four different methods were tried.

2.2.1.3.1 Wet etching of fused silica

Fused silica is etched isotropically by hydro fluoric acid (HF). To make curved geometries we fabricated samples with sharp edges (Section 2.3.1.2). These substrates were wet etched for different times in 6:1 buffered oxide etch (BOE) – a milder form of HF. Isotropic etch should etch the geometries in all directions at an equal rate, but it did not. The top corners of the substrates etched at a higher rate that the bottom corners rendering the bottom corners curved but the top edges remain sharp as shown in Fig 2.9. This occurs because the isotropic etching front proceeds as a spherical wave from all points open to the etchant, hence the bottom corners of ridges get curved and the top corners are etched with a higher rate. An appropriate analogy will be machining a substrate on a milling machining, where the tool is unable to reach the bottom corners and thus never gives a sharp bottom corner. Thus, this method was not used.

![Figure 2.9. SEM images of fused silica etched using 6:1 BOE (a) 15 min etch (b) 8 min etch.](image)

Starting width of samples in both cases was 1.5 μm. Imaged on a 45° stage.
2.2.1.3.2 Wet Etching of Silicon Di Oxide

To address the above problem we decided to make the inverse geometry in SiO₂ and mold PDMS to get the substrates with a controlled radius of curvature. Fig. 2.10 shows the schematic of the fabrication process. Photoresist was spun of four-inch silicon wafers that had a 1.4 μm thick silicon dioxide film. The resist was exposed and developed using process mentioned above (Section 2.3.1.2). With photoresist as mask SiO₂ was etched to a certain depth using a fluorine based chemistry and the etched samples were immersed in a 6:1 solution of BOE for isotropic wet etch. The samples were removed and washed with DI water followed by an exposure to O₂ plasma to strip the resist.

![Diagram of fabrication process]

Figure 2.10. Schematic of the fabrication process for curved substrates using SiO₂ wet etch.

Scanning electron microscopy of the samples revealed that wet etch left the surface rough (Fig. 2.11c, d), thus unsuitable for experiments.
Figure 2.11. SEM images of exposed and etched samples. (a, b) Samples etched to 300 nm in fused silica with resist as mask. (c, d) Sample in (a) was etched for 60 s in 6:1 BOE to give 100 nm radius of curvature. Images taken on a 45° stage.

### 2.2.1.3.3 Resist reflow technique

This method is used extensively for the fabrication of microlenses [17]. The principle behind this technique is that most resists are amorphous polymers that exhibit viscous flow with considerable molecular motion of the polymer chain segments above the glass transition temperature ($T_g$) and at temperatures below $T_g$, the motion of the segments is halted, and the polymer behaves as a glass. When heated above $T_g$, the polymer flows easily, thus heating the resist film above its $T_g$ for a reasonable time enables the resist to take shape that has curved edges [18]. Fig. 2.12 shows the schematic of the fabrication process. A silicon
master was fabricated using the technique in Section 2.3.1.2. 5 μm thick layer of PMMA 950 A11 was spun on glass cover slips, using nanoimprint lithography the master is brought in contact with a mold, applying a high pressure and a temperature higher than the glass transition point (Tg) of the polymer, so that the polymer material reaches a viscous flow state [19]. The 3D features on the Si master surface penetrate the molten polymer film, giving the film surface a 3D shape that is a reverse image of the master. The imprinted resist was then heated on a contact hot plate at 190° C for 5 min. AFM scans of the molds revealed the change in resist profile pre and post resist reflow shown in Fig. 2.12 (e,f). The drawback of this method was a difficulty in the precise control of feature dimensions.

![Diagram of fabrication process](image)

**Figure 2.12.** Schematic of the fabrication process.
2.2.1.3.4 Fabrication of curved substrates in fused silica using spacer technique

This technique is a slight modification of fabrication of sidewall spacers [20] in semiconductor industry. Because of the need for smaller initial line widths, high-resolution OIR 620-7i resist was used. The resist was patterned and developed as above. Samples with line widths from 500 nm – 1.2 μm were generated. The samples were etched to two heights, 200 and 400 nm, followed by resist strip and cleaning. Fig 2.13 shows the developed resist and etched fused silica pattern. Finally, SiO\textsubscript{2} was deposited on the features by plasma assisted chemical vapor deposition (PECVD) with gases N\textsubscript{2}, SiH\textsubscript{4} and N\textsubscript{2}O at 400\degree C. This process results in conformal deposition and rounding of the features to a controlled radius of curvature approximately equal to the thickness of the deposited oxide. Fig 2.14(a) shows the schematic of the fabrication process and Fig 2.14 (b-e) show cross-sectional SEM images of substrates with h = 400 nm. The samples have constant w = 1.3 μm and r varying from <10 nm to 400 nm. AFM scans are shown in Fig 2.15. Using this technique we were able to fabricate substrates with precise radius of curvature, moreover it was repeatable over time and these substrates were used for studying the effect of curvature on cellular mechanotransduction.
Figure 2.13. SEM images of sub micron features that were coated conformally with oxide.

(a) Exposed resist. (b) Etched fused silica. SEM images taken on a 45° stage.
Figure 2.14. Fabrication of substrates with controlled radius of curvature (r). (a) Schematic of the microfabrication process used to make the features with a given r. Drawings not to scale. (b,c,d,e) Cross-Section scanning electron micrographs of substrates with varying r, from 0, 100, 200 and 400nm. (Note that other dimensions like ridge width (w), pitch (p) and height (h) of the features is same in all the substrates). Scale Bar – 1 micron.
Figure 2.15. AFM measurements of samples pre and post oxide deposition. (a) Pre deposition height of features - 400 nm. (b) Post deposition height of features – 400 nm.

2.2.2 Surface Characterization

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) was used to characterize the substrates. The dimensions analyzed with SEM were the periodicity of the structures i.e. pitch, ridge and groove width whereas for height characterization AFM was used. The samples have constant $w = 1.3 \, \mu m$, $p = 3 \, \mu m$ and $r$ varying from $<10 \, nm$ to $400 \, nm$. 
2.2.3 Cell Culture

Silanized samples coated with 99.9% Hexamethyldisilazane (Sigma – Aldrich) were coated with human plasma fibronectin (10 μg ml⁻¹; Roche) for 1.5 hours at 37 °C and 5% CO₂. Mouse Embryonic Fibroblasts (MEF) were maintained in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 1% L-glutamine and 100 IU/mg penicillin-streptomycin (Invitrogen) at 37°C and 5% CO₂. MEFs were plated 24 h before an experiment at 80,000 cells per 1.5 cm² tissue culture dish, harvested and added to substrates at 100,000 cells/ml in ringer solution (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM Hepes and 2 g/l glucose, pH 7.4). Each of the petri dishes was filled with 2.7 ml of ringer solution and 0.3 ml of cells in ringer, i.e. 30,000 cells were seeded on the substrate. Cells from passage 12 – 17th were used during the experiments.

2.2.4 Immunofluorescence Staining

Fibroblasts were seeded onto the substrates. On the first set of substrates (sharp features with varying heights), the cells were allowed to spread for 75 min. On the second set of substrates (variable r) the spreading time was 150 min. After the spreading period, the cells were fixed with a solution of 4% paraformaldehyde in PBS preheated to 37 °C for 10 min. After fixation the samples were treated with a blocking/permeabilizing solution of 1% BSA/PBS for 1 hour at room temperature followed by the addition of anti paxillin (B.D. Biosciences) and anti tubulin (gift from Prof. Chloe Bulinski) primary antibody (1:500 in 1% BSA/PBS) for 2 hours. Next the samples were rinsed for 5 min three times with PBS followed by a 5 min rinse with antibody dilution buffer. After this the substrates were stained overnight with secondary antibody for paxillin (AlexaFluor 488 Invitrogen; 1:500 in 1% BSA/PBS) and microtubules (AlexaFluor 555 Invitrogen; 1:1000 in 1% BSA/PBS), additionally cells were also stained for F - actin with AlexaFluor 633 phalloidin (Molecular
Probes). Phalloidin labeling was performed together with the secondary antibody labeling. Each of the samples was then rinsed for 5 minutes with PBS four times. Finally the substrates were coated with 100 µl of ProLong Gold Antifade Reagent (Invitrogen) to suppress photobleaching. Stained cells were imaged using a 40 x, 1.35 NA oil objective (Olympus). An average of 20 – 40 and 50 – 70 cells were analyzed on each of the sharp and curved substrates respectively.

2.2.5 Time-lapse microscopy of live cells

Differential interference contrast (DIC) microscopy was used for live observation of cells on the patterned substrates. The substrates were glued onto the bottom of the 35 mm falcon petri dish with a hole in the center. Time-lapse micrographs were recorded with a 20 x, 0.7 NA air objective (Olympus) through a cooled CCD camera CoolSNAP HQ (Roper Scientific Inc.) using Simple PCI software (Compix Inc.). Images were captured every 5 seconds.

2.2.6 Cell-to-substrate interaction using Scanning Electron Microscopy

SEM analysis was performed to determine whether the cells were lying on top of the features or conforming to the patterns. For this analysis, the cells were fixed using 0.1% glutaraldehyde (Calbiochem) for 60 seconds. After fixation the cells were dehydrated in a graded series of chilled ethanol (50%, 60%, 70%, 90% and 100%) and then critical point dried with liquid CO₂. Dried substrates were sputter coated with 10nm Au-Pd and observed using SEM (Hitachi 4700) at an accelerating voltage of 5 kV. In both cases i.e. substrates with zero and controlled radius of curvature the cells were on the ridges and did not dip into the grooves.
2.3 RESULTS

2.3.1 Fabrication of substrates with controlled radius of curvature (r)

We have developed a fabrication technique to precisely control the radius of curvature of features. Using this technique two sets of samples were prepared with the following dimensions.

(i) \( h = 200\text{nm}; r = 0 \) (‘sharp’), 50, 100, 200 nm, \( r = \infty \) (flat).

(ii) \( h = 400\text{nm}; r = 0, 50, 100, 200, 400 \) nm, \( r = \infty \).

In all samples the feature width was 1.3 \( \mu \)m, close to the size of a single focal adhesion and the pitch was 3 \( \mu \)m. We note that the nominal value of \( r = 0 \) (‘sharp’) substrates is for samples with only the as-patterned features, which have a true radius of curvature below 10 nm. The geometry of the substrates was verified using SEM (width and pitch) and AFM (height). This method of controlling \( r \) is very precise and reproducible with no more than \( \pm 10\% \) error. Additionally, the area patterned with oxide is surrounded by planar smooth area covered with the same material, allowing for analysis of cellular behavior in a control condition and on topographic features simultaneously. This is the first demonstration of the fabrication of structures in which radius of curvature is independently controlled on the nm scale, for the study of its effect on cellular mechanotransduction.

2.3.2 Analysis of cell morphology on sharp substrates

The analysis of cell morphology was done in 2 steps. First, cells were spread for 75 min on sharp substrates with varying heights (50, 100, 200, 400, 600, 800 and 1000 nm). This time period is well beyond the stabilizing phase of spreading, which is around 20 min [21]. This allowed sufficient time for the cells to stabilize from the active spreading phase [confirmed by live microscopy]. The shape of the cells was determined by drawing a best-fit ellipse around the cells using a standard Image J plugin [National Institutes of Health (NIH)]. The
degree of cellular polarization was quantified by calculating the anisotropy ratio (AR), defined as the ratio of length of the cell parallel to the features to the length perpendicular to the ridges. As shown in Fig 2.16, the AR is close to 1 up to \( h = 100 \) nm, then increases to a value of \(~3-4\) for \( h = 400 \) nm, above which it is roughly constant. Therefore we identify \( h = 100 - 200 \) nm as the threshold for contact guidance in this system. The observed response to sharp substrates was in accordance with previous studies [16, 22, 23] where it has been reported that AR increases with the height of grooves and ridges. For prolonged spreading it has been reported that the threshold height for alignment is in \( 35 - 75 \) nm range [8]. We note that the AR also increases with decreasing feature width [24], although this parameter was not examined here.

![Graph](image)

Figure 2.16. Effect of feature height on cell shape. Error bars indicate mean +/- Standard Deviation.
2.3.3 Analysis of cell morphology on curved substrates

Based on the above results, samples with $h = 200$ and $400$ nm, and varying $r$, as described above, were used to examine the effects of curvature. Using these substrates, we investigated the effect of curvature on cell area and AR (Fig. 2.17). The spreading time on these substrates was 150 min, which allowed sufficient time for the formation of focal adhesions. On both heights, the spread area increases with $r$, and is higher on flat substrates than on substrates with patterned lines (Fig. 2.17a, b). On 200 nm high features, the spreading area showed an increase from 2500 $\mu$m$^2$ to 4000 $\mu$m$^2$ as $r$ increased from $\sim 0$ to 200 nm. On 400 nm high features the spreading area showed an increase from 2400 $\mu$m$^2$ to 3400 $\mu$m$^2$ as $r$ increased from $\sim 0$ to 400 nm. One-way ANOVA tests revealed that both of these changes were statistically significant ($p < 0.001$).

For both 200 nm and 400 nm feature heights, the AR decreased smoothly with increasing $r$. On 200 nm high substrates, the AR decreased from 3.3 on sharp features to 1.7 on features with $r = 200$ nm. On 400 nm high features, the AR decreased from 4.8 on sharp surfaces to 2.4 on features with $r = 400$ nm. These changes were statistically significant ($p < 0.001$). The AR was 1.2 on flat substrates. Thus, increasing the radius of curvature, while maintaining the feature height, can cause the cells to assume a shape that is close to that observed on flat substrates. For 400 nm high features the AR decreases smoothly with radius of curvature up to $r = 200$ nm, after which the change is statistically insignificant ($p > 0.75$).

Interestingly, 400nm high lines consistently ($p < 0.001$) showed larger AR than the 200 nm high features, at all values of same $r$. This observation raised the possibility that cells might contact the troughs of the grooves. To ensure that the cells are not dipping inside the grooves, scanning electron microscopy was used to precisely examine cellular morphology on the patterns. It was observed that cells attach themselves mainly to the top of the ridges and do not descend into the grooves, as shown in Fig. 2.18. This observation suggests that
the radius of curvature of the tops of the ridges is what the cells are primarily sensing. Nevertheless, given the slightly greater polarization on the taller lines we cannot rule out minor contacts with the base of the features.

Figure 2.17. Spread area and anisotropy ratio vs. radius of curvature. (a, b) Cell Spread area vs radius of curvature on 200 and 400 nm high substrates respectively. (c, d) Anisotropy ratio vs radius of curvature for 200 and 400 nm high substrates respectively. Asterisk indicates statistical significance relative to r \sim 0 \text{ nm} \ (p < 0.001). Error bars indicate mean +/- Standard Deviation.
Figure 2.18. Cells spread on top of features. Scanning electron micrographs of cells on grooves and ridges with different radius of curvature. (a, b) MEF cells spreading on substrate with $h = 400$ nm and $r \sim 0$ nm. (b) An enlarged section of the image in panel (a) showing the cell lying on the ridges. (c, d) Cell spreading on substrate with $h = 400$ nm, $r = 400$ nm. Cells were fixed after 2h 30 min and critical point dried. Cell marked with asterisks and substrate marked with arrows (white – ridges, black – grooves).

Scale Bars - a and c, 30 μm; b and d, 5 μm.
Figure 2.19. Cells stained for actin (blue), tubulin (red) and paxillin (green). (a) Cell on a substrate with $h = 200\text{nm}$, $w = 1.3\ \mu\text{m}$, $p = 3\ \mu\text{m}$ and $r < 10\text{nm}$. (b) Cell on substrate with same $h$, $w$ and $p$ as (a) but $r = 200\ \text{nm}$. (c) Cell on flat substrate. Cells on sharp substrates align and elongate along grooves and ridges, cells on flat substrates are mostly round where as cells on substrate with a radius of curvature show a morphology in between that of sharp and control substrate. Cells were spread for 150 min. Arrow indicates the direction of pattern. Scale bar - 20 $\mu\text{m}$.

### 2.3.4 Cell cytoskeleton and focal adhesions

Fluorescent observation after 150 min of cell spreading revealed well-defined actin fibers and microtubules (Fig. 2.19). On the patterned substrates, the stress fibers and microtubules were polarized along the lines, whereas on a flat surface no such polarization was observed. On all substrates, microtubules originated from the microtubule organizing center (MTOC, near the nucleus), out to the cell periphery. The microtubules on both the sharp and curved substrates were partially aligned in the direction of pattern as shown in Fig. 2.20(b,e). On flat surface the cells took an isotropic shape with round stress fibers at cell periphery and diffused actin in the cytoplasm as shown in Fig. 2.20(g). On flat substrates microtubules seem to form a radiating network from the MTOC close to the nucleus to the outer edge of cell as shown in Fig. 2.20(h). On flat control surface numerous
focal adhesions were found all around the circumference of the cell as shown in Fig. 2.20(i). Where as on sharp and curved surfaces the focal adhesions formed in the interior of the cell, on top of ridges (Fig 2.20 c,f). On sharp and curved surfaces the nuclei of the cells were aligned along the pattern where as on flat surface a mix of randomly elongated and rounded nuclei were observed.

Interestingly, although cells on the r = 200 nm and 400 nm substrates had spreading areas and polarization that resembled flat substrates (Fig 2.17), these substrates nevertheless displayed robust focal adhesions and cytoskeleton alignment to the grooves (Fig 2.20 a,c,d,f).
Figure 2.20. Fluorescent images of cytoskeleton of fibroblasts on sharp, curved and flat substrates after 150 min of spreading. On substrates with $r \sim 0$ nm (a) the stress fibers and actin are polarized. (b) Tubulin is aligned in the direction of grooves and ridges. (c) Focal adhesions were aligned along the ridges in the protruding lamellipodium. Fibroblasts on pattern with $r = 200$ nm exhibit a morphology between cells on substrate with $r \sim 0$ nm and flat surface, (d) Stress fibers were aligned in the direction of grooves and ridges and the actin network was well organized. (e) Microtubules were polarized in the direction of pattern. (f) The cells had significantly higher number of aligned focal adhesions as compared to cells on substrates with $r \sim 0$ nm. Cells on flat surface. (g) Actin cytoskeleton was well developed with circular bundles of stress fibers throughout the cytoplasm. (h) Microtubules in cells were well organized. (i) Focal adhesions were seen around the periphery of the cell. Arrow indicates the direction of grooves and ridges.

Scale bar - 20 μm.

2.4 DISCUSSION

The present study is the first examination of the role of substrate curvature in contact guidance where the radius of curvature is varied independently of other dimensions. We found that for 1.3 μm wide grooves separated 3 μm apart, features needed to be higher than 200 nm and their radius of curvature below 200 nm to evoke robust morphological responses. However, cytoskeletal and adhesive effects were seen at radii of curvature as high as 400 nm.

It has been shown that after 24 hours, fibroblasts can reorient to grooves as small as 35nm deep [8]. On our substrate, cellular morphologies to feature heights of 100 nm and lower approximated a flat surface, with an AR of approximately 1.5 (Fig 2.16). Two important differences from the previous study reporting greater sensitivity were the length
of culture (24 hours versus 75 minutes in this study) and the width of the grooves (100 nm versus the 1.3 μm in this study). We investigated the effect at shorter times to better understand the effect of r on features 200 and 400 nm high due to the sharp transition in the polarization values (AR) at these two heights. We note that this range is similar size to the size of ECM fibrils [15] in vivo.

These results can help to clarify the contribution of various mechanisms in contact guidance. It has been proposed that the frequency of filopodia formation in the direction perpendicular to the features is lower because of the stress involved in bending the filopodia around a sharp corner, leading to cell polarization [25]. Although the cells in these studies more commonly show a smooth leading edge (lamellipodium), curvature should modify the protrusion of lamellipodia in the same way as filopodia. These results suggest that bending stress begins to modify protrusion below a radius of curvature of ~200 nm, a prediction that can be directly studied in further experimental and theoretical work. A second proposed mechanism is confinement and alignment of focal adhesions to the top surfaces of patterns: focal adhesions orient themselves to the pattern, which in turn polarizes the actin filaments that originate from the focal adhesions, and thus the entire cell [26]. However, we observe that focal adhesions remain confined to ridges even for large r, where cell spreading is close to isotropic. Therefore, these results suggest that this mechanism alone cannot cause cell polarization.

Finally, the range of curvature sensing seen in this study will help to determine whether membrane-bound curvature-sensing proteins are involved in contact guidance. For example it has been reported that BAR (Bar/Amphipysin/Rvs) domains are the sensors of membrane curvature [27-29]. Recently, Bhatia et al [30] examined eNBAR proteins on structures with varying radii of curvature and found monotonic drop-off in binding up to r ~ 100 nm, after which the decrease slows. This cutoff in curvature is somewhat smaller
than seen in our sample, indicating that different sensing mechanism may be at play here. However, the difference is not so large as to definitively rule out this mechanism.

Our findings are relevant for tissue regeneration. For a tissue to function/regenerate properly it is necessary that the building blocks i.e. individual cells be healthy. It has been reported that for individual cells to survive they need to have large spread area [31] and for a tissue to elongate polarized stress fibers are required [32]. The cells on the curved surfaces show both these conditions, and thus curved geometry could potentially be applied in designing better implants.

2.5 CONCLUSION

This is the first study where the radius of curvature was controlled precisely at the nanometer scale. In this study we demonstrated the fabrication process of a substrate with controlled radius of curvature from 50 nm to 400 nm. The features were fabricated in fused silica, which made sure that the cell response was due to the underlying geometry and not because of change in rigidity; which might be the case with elastomeric substrates. Furthermore early spreading (75 min) results reveal that the threshold height sensed by the fibroblasts for sharp substrates with groves and ridges, 1.3 μm wide and 3 μm in pitch was in the 100 - 200 nm range. Additionally, we also showed a definitive influence on cellular behavior and morphology due to controlled curvature and cells are able to sense and respond to radius of curvature over a wide range 50 – 200 nm. The potential application might be in the area of tissue engineering in particular where independent control of cell morphology and fiber alignment is required. Future work will be targeted at exploring the mechanisms that lead to curvature sensing by fibroblasts.
REFERENCES


Chapter 3

3.1 INTRODUCTION

Mechanical factors such as force, geometry and rigidity play a critical role in various biological processes, including cell migration, adhesion, cytoskeletal reorganization and cell differentiation [1-5]. Cells sense mechanical forces and respond by converting them into biochemical signals through a variety of proteins and protein complexes. Investigation and understanding of the forces exerted by the cell on its environment is crucial in understanding a variety of physiological processes. For example, a change in the force a cell exerts on a surface could be an indication of disease; it has been reported that cancer cells exhibit larger force than the normal cell by ~ 20% - 50% [6].

The main force-generating structure in cells, the actomyosin cytoskeleton, can be thought of as exerting two types of forces, traction and protrusive forces. Traction forces are the forces exerted by the cell to a substrate (or another cell), as the cell moves on it or simply attaches to it. The major single component generating this force is the contraction of myosin on actin filaments [7]. Where as protrusive forces are a result of actin polymerization at the leading edge of the cell that drive lamellipodial movement and cell migration [8].

Recent advances in microfabrication have enabled direct observation and quantitative analysis of force generation at the single cell level. Traction forces have been measured using a variety of methods, such as silicone rubber membrane, micropipettes, optical tweezers, nanowire arrays, and elastomer micropillar arrays [9-13]. Elastomeric pillars are particularly useful for this purpose, since they allow the mapping of forces
applied by a cell at the micron level directly from measurement of the deflections of an array of flexible pillars to which the cell attaches [13]. Elastomeric pillar devices have been used to measure traction forces applied by several cell types [14, 15].

The study of protrusive forces, however, has been much more elusive. For this measurement, fluid flows [16], Atomic Force Microscopes [17], or glass fibers [18] have been used to stall the movement of highly migratory keratocytes, and subsequently measure or model the force. These studies found effective lamellipodium polymerization pressures of $\sim 1$ kPa. However, these experimental approaches have the problem of fundamentally altering the state of the cell (by stalling its movement), being limited to one cell at a time, and not being able to map force generation in different points of a cell. Furthermore, they all require specialized and complex equipment attached to a biological microscope.

To solve these problems, in this study we present the development and use of a microfabricated device to measure the protrusive force exerted by NIH 3T3 fibroblasts during spreading. The device consists of a continuous substrate with rows of micron sized pillars that are a little taller and right next to 250 μm wide pads (Fig. 3.1). The pillars are 150 nm taller than the pads i.e. for pillar length 5 μm the height of pads is 4.85 μm. Since protrusive forces are a result of actin polymerization at lamellipodia – a broad, flat sheet-like structure, with a thickness in the range of 100 – 200 nm [19] this difference in height ensures that lamellipodia encounters the pillars and deflects them.

The device comprises multiple, independent force sensors that are available for analysis. Deflection of pillars is tracked using bright field microscopy and force is calculated using Hooke’s law. The force displacement relationship for a tapered cylindrical beam is:

$$F = \left(3\pi Ed_b d_s^3 / 64h^3\right) \Delta x$$

(1)
Where $E$, $d_o$, $d_b$, $h$, $D_x$ are the Young’s Modulus, tip diameter, base diameter, height and deflection of the pillars respectively.

![Diagram of force sensor device in silicone elastomer (PDMS).](image)

Figure 3.1. Force sensor device in silicone elastomer (PDMS). (a) Top view where pillar diameter ($d$) = 1µm, distance between edge of pad and pillar ($d_1, d_2$) = 2, 1µm respectively, protrusive forces were measured on the block of pillars where the cell first makes contact i.e. the pillars to the right of the dashed line. (b) Cross section view. (c) Magnified view of the area in black square, dashed lines indicate the height difference between pads and pillars. Cell spreading on the pads encounters the pillars and deflects them. Note the height of pillars is higher than the nearby pads.

Forces are computed using Hooke’s law. Fig. 3.2 shows deflection of the microposts, this is tracked using bright field microscopy and multiplying it by the spring constant of pillars gives the force.
Figure 3.2. Schematic of a deflected pillar of diameter (d) and height (h). Force (F) applied at the pillar tip results in deflection (Δx).

3.2 EXPERIMENTAL

3.2.1 Fabrication

Since the device in Fig. 3.1 is made of an elastomeric material (polydimethyl siloxane, PDMS), a rigid mold must be formed which has different depths for the pads and pillar arrays, respectively. Fabrication of the mold was performed in three major steps: (1) fabrication of the holes with a head start in Si, by photolithography and etching; (2) fabrication of the pads around the holes; (3) etching of the entire pattern with holes and pads, to the desired depth which leaves the holes 100 – 200 nm deeper than the pads. The sequence of microfabrication is illustrated schematically in Fig. 3.3 and described in detail below.
3.2.1.1 Fabrication of holes with head start

Four-inch silicon wafers were subjected to a standard RCA clean, followed by thermal oxide growth. The wafers were oxidized at 1100 °C for 4.5 hours to form a 1.4 μm thick silicon dioxide (SiO₂) film that acts a hard mask for a Si etch. To promote adhesion between the wafer and the photoresist HMDS was applied to the wafers, after which the substrates were spin-coated with 1.2 μm-thick photoresist (Shipley SPR 700). Following resist deposition, the wafers were soft-baked on a hot plate at 90 °C for 60 s to drive residual solvents away from the photoresist film. The wafers were patterned by conventional UV photolithography. A photomask, with hexagonal array of holes, for the first level of lithography was formed using a Heidelberg DWL 66 laser pattern generator. The photoresist was exposed for 170 ms and patterned with a 5:1 i-line (365 nm) reduction stepper (GCA Autostep 200). After exposure the wafers were postbaked on a hot plate at 115 °C for 60 s and developed for 60 s with AZ 200 MIF developer, followed by an O₂ plasma descum for 45 s. Development of a finely tuned etch recipe was a critical part of this fabrication process as the device was required to have smooth, vertical side walls and flat bottom, which is essential for the release of polydimethylsiloxane (PDMS) from the Si mold. Adjusting the gas ratios, pressure and power was challenging as two different patterns; with size difference of two orders of magnitude were being etched simultaneously. For Si etch two different etch chemistries were tried, a chlorine based etch and a fluorine based etch and a fluorine based etch gave molds with minimum defects and these were used to cast PDMS.
Figure 3.3. Schematic diagram illustrating the fabrication of force sensor in PDMS with pillars higher than nearby pads.

### 3.2.1.2 Etching of molds with Chlorine based etch chemistry

In this approach Si was etched with oxide as hard mask and PlasmaTherm 770 inductively coupled plasma ICP - RIE system was used. Si was etched in a chlorine based ICP-RIE system under various conditions involving different changes in the gas ratios, pressure and power. The gases used were Boron trichloride (BCl₃), Chlorine (Cl₂0, and Hydrogen (H₂). Numerous tests were run with all the parameters but the results obtained were not good. This made us investigate a few things. First, the skewed profile may be a result of bad silicon oxide (SiO₂) etch, to address this issue electron micrograph of the samples after oxide etch was taken.
(Fig. 3.4a). It can be clearly seen that the oxide etches fine with vertical sidewalls and a clean bottom surface. Furthermore, to know when exactly does the deterioration of profile in Si occurs the samples were imaged after 90 sec short plasma etch. It was observed that the holes start taking a tapered profile right form the start of etching (Fig. 3.4b).

Figure 3.4. (a) SEM of etched holes in oxide. (b) Brief silicon etch with oxide as mask. Grains in (a) are due to gold sputtering.

Thus nothing was wrong in the oxide etch recipe being used. Fig. 3.5 shows the different etch profiles obtained, it can be seen all of the profiles either had a tapered surface it the bottom was not flat. Flat bottom is important as for biological experiments silicone elastomer was to be molded and if the bottom is not flat then the elastomer does not peel off smoothly.
Figure 3.5. SEM images of silicon etch using chlorine based chemistry. (a) Holes have a tapered profile and pads are not flat (b) Both the pads and holes show kinks at the edges. (c) Bottom surface of the holes is not flat. (d) Top part etches more than bottom leading to a funnel shaped profile.

3.2.1.3 Etching of molds with fluorine based etch chemistry

This recipe was optimized for giving the desired results i.e. flat bottom and smooth sidewalls. The photoresist pattern was transferred into the oxide using reactive ion etching (RIE) (Oxford PlasmaLab 80+). For the characterization of this recipe it was important to calculate the selectivity of the mask, as if the mask is not thick enough the resultant profile is affected (Fig. 3.6). Running various tests it was found that the selectivity of silicon to silicon di oxide was 6:1.
Figure 3.6. SEM of holes with inadequate mask for etch. (a) Etch grass seen on the top surface. (b) Holes take a wider shape at the top.

Prior to Si etch, using resist as mask oxide was etched with flow rates of 2 SCCM (SCCM – standard cubic centimeter per minute) O$_2$, 50 SCCM CHF$_3$, 40 mTorr pressure, and 200 W rf power, etch rate of SiO$_2$ was measured to be around 30 nm/min. A 20% over etch was applied to make sure that all of the SiO$_2$ is gone. Next the wafers were etched using Unaxis 770 Deep Si Etcher. This step gives the holes a head start over the pads, which are fabricated in the second step. Resist was not stripped because it acts as a mask for Si etch. If all of the resist is consumed during etching the oxide layer acts as a hard mask for etch. Si was etched with a 3 step fluorine etch process. Gas stabilization was the first step, followed by lighting up of plasma and etching of the native oxide for which the conditions were: 40 SCCM Ar, 10 SCCM O$_2$, 40 SCCM C$_4$F$_8$; 8 m Torr pressure; and 35 W rf power and 700 W ICP power. The third step etches the Si substrate and hence its duration determines the amount of head start to the holes. The gas flow rates were 40 SCCM Ar, 10 SCCM O$_2$, 60 SCCM C$_4$F$_8$, 30 SCCM SF$_6$; with 8 m Torr pressure; and 10 W rf power and 700 W ICP power. Etch time was adjusted in accordance to the difference between the depth of holes and pads as this difference had to be in the 100 – 200 nm range. The tolerance was very small and a lot of
etch runs were made to get the desired results. Fig. 3.7 shows the etched samples where the difference in height is not in the 100 – 200 nm range.

Figure 3.7. SEM images of samples showing the holes higher than pads. (a) 300 nm (b) 1.2 μm (c) 430 nm (d) 630 nm. Samples were etched using fluorine based chemistry and all samples had a flat bottom and straight sidewalls for both pads and holes.

The next fabrication process involved second level of lithography. For this the wafers were coated with photoresist (SPR 220 – 3) and soft-baked on a hot plate at 115 °C for 90 s. The resist was then exposed in a stepper for 190 ms using a mask that had 250 μm wide pads and immediately post exposure baked at 115 °C for 90 s. Exposed portions of the resist were developed with AZ 300 MIF developer. Next the substrate was descummed followed by an oxide etch. After this the photoresist was stripped using O2 plasma. The area
around holes and pads covered with SiO\textsubscript{2} acts as a hard mask for the final etch step. The same etch recipe as mentioned above was used to etch the pattern. Etch rate of Si for holes and pads was 200 nm/min and 373 nm/min respectively. After pattern transfer into the silicon substrate, the oxide hard mask was removed by a 5 min immersion in 6:1 buffered oxide etch (BOE). Holes were etched for a total of 25 min (12 min in first step and 13 min in the last step) and pads etched for 13 min.

![Cross-sectioned scanning electron microscope (SEM) image of force sensor in the silicon mold after fabrication. The holes are ~150 nm deeper than nearby pads. Stage tilt: 90 degree. Scale bar ~ 1 μm.](image)

Molding PDMS into the master gives the reverse structure, which is an array of posts and rectangular pads with bottom surface at same level but the top surface of posts 150 nm higher than pads (Fig. 3.8).
3.2.2 Calibration of spring constant

In the linear bending regime, the deflection of pillars is directly proportional to the force. Within the validity range of Hooke’s law, the force displacement relationship for a cylindrical beam is:

\[ F = \left( \frac{3\pi E d^4}{64h^3} \right) \Delta x \]

Where E, d, h, Δx are the Young’s Modulus, diameter, height and deflection of the pillars respectively. Since the posts used were not a perfect cylinder, but tapered with bottom diameter at d_b and top diameter as d_t, shown in the Fig. 3.9, spring constant for this geometry had to be calibrated.

Assuming a force F acts on the free end of the pillar. According to the elastic curve equation:

\[ \frac{d^2x}{dy^2} = \frac{M}{El} \]  \hspace{1cm} (2)

where M = bending moment

I = Moment of inertia

E = Young’s modulus

M = - Fy  \hspace{1cm} (3)

I of perfect cylinder with diameter d and circular cross section

\[ I = \frac{\pi d^4}{64} \]  \hspace{1cm} (4)

As shown in Fig. 3.9 the pillars on the device are tapered, I at a distance y from tip is:

\[ I = \frac{\pi}{64} \left( d_t + (d_b - d_t) \frac{y}{h} \right)^4 \]  \hspace{1cm} (5)

d_t = tip diameter

d_b = base diameter
$h = \text{pillar height}$

**Figure 3.9.** Tapered pillar with different diameters of base and tip. Due to difference between tip and base diameter the effective spring constant of pillars changes.

Substituting $M$ and $I$ from eq. (3) and (5) in eq. (2)

$$\frac{d^2x}{dy^2} = -\frac{64F}{\pi E} \frac{y}{\left(d_t + (d_b-d_t)\frac{y}{h}\right)^4}$$

(6)

Let

$$k = \frac{64F}{\pi E}$$  \hspace{1cm} (i)

$$a = d_t$$  \hspace{1cm} (ii)

$$b = d_b$$  \hspace{1cm} (iii)

Substituting in eq. (6)

$$\frac{d^2x}{dy^2} = -k \frac{y}{\left(a + (b-a)\frac{y}{h}\right)^4}$$

(7)
Chapter 3. Measurement and Mapping of Protrusive Forces in Fibroblasts

Integrating above eq.

\[
\frac{dx}{dy} = \frac{k h^4}{6(a-b)^2} \left( \frac{a(h-3y)+3by}{(a(h-y)+by)^3} \right) + c_1 
\]  

(8)

Solving eq.8 for \( c_1 \) using the boundary conditions: \( y = h, \frac{dx}{dy} = 0; \)

\[
c_1 = \frac{k h^2}{6b^3} \left( \frac{2a-3b}{(a-b)^2} \right) 
\]  

(9)

Integrating

\[
x = \frac{k h^4}{6(a-b)^3} \left( \frac{2ah-3ay+3by}{(a(h-y)+by)^2} \right) + yc_1 + c_2 
\]  

(10)

Using boundary conditions: \( y = h, x = 0 \) and solving eq.10 for \( c_2 \);

\[
c_2 = \frac{k h^3}{6b^3} \left( \frac{6ab-6b^2-2a^2}{(a-b)^3} \right) 
\]  

(11)

Substituting \( c_1 \) and \( c_2 \) from eq. (9) and (11) in eq. (10) and applying boundary conditions:

\( y = 0, x = \Delta x; \)

\[
\Delta x = \frac{-kh^3}{3ab^3} 
\]  

(12)

\( \Delta x \) = deflection of the pillar tip

Substituting \( k, a, b \) from eq. (i), (ii) and (iii) in eq. (12)

\[
\Delta x = -\frac{64Fh^3}{3\pi Ed_d d_b^5} 
\]

Comparing the above equation with Hook's Law i.e. \( F = -k\Delta x \), the revised spring constant is:

\[
k = \frac{3\pi Ed_d d_b^3}{64h^3} 
\]  

(13)
3.2.3 PDMS Molding and Surface Chemistry

The patterned wafers were cleaned in piranha (a mixture of 70% sulfuric acid and 30% hydrogen peroxide by volume) for 2 hours at room temperature followed by a 60 s O₂ plasma clean. They were then silanized in vacuum with vapor phase Tridecafluoro-1,1,2,2-Tetrahydrooctyl-1-Trichlorosilane (United Chemical Technologies) for 12 hours to facilitate the release of the elastomer from the wafer post-curing. PDMS (Sylgard 184, Dow Corning) was mixed thoroughly with its curing agent in a 10:1 ratio and degassed in a vacuum chamber for 10 min to remove trapped air. It was poured over the Si mold and cured at 65 °C for 12 h. The modulus of elasticity (E) of PDMS was measured using 8 mm cylindrical PDMS specimens of thickness 2.24 ± 0.16 mm. Prior to this the PDMS was baked in 60 mm petri dishes for 12 h at 65 °C. The tests were conducted on an Instron machine with a 2 kN load cell, extension rate of 5 % s⁻¹ and 20% maximum strain. A linear stress-strain relationship was used with the force-strain data to determine the modulus of elasticity of PDMS as 2.51 ± 0.39 MPa. The PDMS was then peeled off the silicon mold while immersed in pure ethanol. Use of ethanol is critical to this step as its low surface tension wets the pillar array, thus avoiding collapse of the pattern. After this the PDMS substrates were transferred onto a silanized glass cover slip and placed in a 35 mm petridish filled with buffer.

3.2.4 Cell Culture

To promote cellular adhesion, the substrates were coated with 10 μg/ml fibronectin in phosphate buffered saline (PBS) for 1 hour at 37 °C and 5% CO₂ prior to plating. Mouse Embryonic Fibroblasts (MEF) were maintained in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 1% L-glutamine and 100 IU/mg penicillin-streptomycin (Invitrogen) at 37°C and 5% CO₂. MEFs were plated 24 h before an experiment at 80,000 cells per 1.5 cm² tissue culture dish, harvested and added to substrates at 100 000 cells/ml in ringer
solution (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM Hepes and 2 g/l glucose, pH 7.4). Before plating the cells, the substrates were washed three times with PBS. Each of the petridish was filled with 2.7 ml of ringer solution and 0.3 ml of cells in ringer i.e. 20,000 cells were seeded on the substrate.

### 3.2.5 Video microscopy and force measurements

The pillars were imaged using an inverted bright field microscope with stage in an isolated, temperature (37° C) controlled box. Time-lapse micrographs were recorded every 3s with a 40X (0.6NA, Olympus) air objective through a CCD camera CoolSNAP HQ (Roper Scientific Inc.) using Simple PCI software (Compix Inc.). Local deformation of the pillars was measured using a particle tracking plug-in for Image J software [National Institutes of Health], which employees an autocorrelation algorithm. The first step is getting an equilibrium (zero force) position, for which the pillars are tracked before the cell deflects them. Second step is the calculation of time-dependent displacement of a given pillar, which is done by subtracting the position of the pillar corresponding to zero force from the position in a given frame. To remove mechanical or thermal stage drift the average displacement of a set of pillars far from any cells was also subtracted from the data. Local forces were calculated by multiplying the deflection of the post and the spring constant of the pillars, which was 5.1 nN/μm, calculated using Eq. 13.

To quantify the protrusion and traction forces we rotated the coordinates (Eq. 14) of the x and y deflections of the pillars. The rotation angle θ was the inclination with respect to the x-axis of a line perpendicular to the cell membrane (measured using Image J). In this way, the y’ value of the transformed coordinates indicated deflection along the axis of the cell membrane. The x’ value indicated deflection perpendicular to the membrane, that is a protrusive force moving away from the cell nucleus (positive values) or a retractive force
moving towards it (negative values) (Fig. 3.10).

Figure 3.10. Matrix rotation for computing the displacement of pillars.

\[
\begin{pmatrix} x' \\ y' \end{pmatrix} = \begin{pmatrix} \cos \theta & -\sin \theta \\ \sin \theta & \cos \theta \end{pmatrix} \begin{pmatrix} x \\ y \end{pmatrix}
\]

\( x' = x \cos \theta - y \sin \theta \)

\( y' = x \sin \theta + y \cos \theta \)
3.3 RESULTS

In this study we measured and mapped protrusive forces in fibroblasts. The main advantages of this device are, 1) it contains multiple force sensors to map forces subcellularly with a resolution of the order of micrometers, 2) by presenting only a minor resistance to the cell, each sensor does not stall or fundamentally alter cell behavior, 3) it is fully transparent and therefore compatible with live measurements in biological inverted microscopes, and 4) simultaneous measurement of both protrusive and traction force with the same device.

3.3.1 Computation of protrusive forces

Protrusive forces were measured by seeding the cells on elastomeric microposts of diameter 1 µm and length of 5 µm with an effective spring constant of 5.1 nN/µm. To avoid the effect of cell-to-cell interaction, cells were plated at subconfluent density. Measurements were carried out for 80 min. As the cells contacted the pillars, a clear increase in protrusive force was observed, which subsided after the lamellipodium went over the pillar. The average maximum protrusive force per pillar, calculated as the difference between the minimum force before the protrusive peak and the maximum point of the peak, was of 1.0 ± 0.23 nN. Different traces represent different pillars on the device with blue color being control, i.e. a pillar situated far from the cell and thus with with zero deflection and force (Fig. 3.11). A few pillars appeared to show a small retraction before protruding (Fig. 3.11d). We believe that this retracting behavior is not the actual pillar deflection but a lensing effect occurring due to a change in refractive index as the lamellipodium moves over the pillar of interest. We verified this by observing a certain change in pillar shape as the cell made first contact with these pillars, that displaced the pillar centroid measured by the tracking software. This time frame corresponded
approximately to the start of the negative dip in Fig. 3.11(d). To compute protrusive forces only the pillars in the first row were analyzed (Fig. 3.12), as these are the pillars that are 150 nm above the pads and lamellipodia of the cell (~150 nm thick) spreading on the pads hits the pillars and deflects them. The peaks of protrusion in the different pillars did not happen simultaneously, emphasizing the heterogeneous and dynamic nature of the lamellipodium.

Figure 3.11. Four representative protrusive force/pillar deflection versus time curves for different pillars. The negative dip in (d) is due to lensing effect and not actual pillar deflection and arrow indicates the moment when the lamellipodia touches the pillar for the first time. The average protrusive force measured was 1 nN. The blue curve in (a) represents the control pillar (pillar with no deflection due to cell hence zero force).
Figure 3.12. Bright field micrograph of cell spreading on PDMS device coated with fibronectin. For calculating protrusive forces pillars on the right side of dotted red line were used as that is where the cell makes first contact. Cell edge has been outlined yellow.

Scale bar - 10 µm

3.3.2 Computation of traction forces

Another interesting observation made was the visualization of traction force on the same device. Once the cell is on top of the pillars it sees the substrates as homogenous array of pillars and exerts traction forces. The average maximum traction force per pillar was computed to be 2.27 ± 0.53 nN (Fig. 3.13). This is approximately twice the protrusive force. Interestingly, the time for traction forces was much higher than the protrusive forces thus pointing out that initially the cell exerts both traction and protrusive forces and after a while the traction forces balance out the protrusive forces.
Figure 3.13. Traction force measurement on device. Traction force/pillar deflection versus time curves for different pillars. Matrix rotation was applied to the X and Y positions of pillars, thus traction forces are negative. Average value of traction forces was 2.27 nN. The blue curve in (a) represents the control pillar (pillar with no deflection due to cell hence zero force).

3.3.3 Computation of pressure exerted by the protruding lamellipodia

Next we calculated the pressure applied by the protruding lamellipodia. Assuming the area in contact with the pillar to be~ 150 nm X 1 μm i.e. the entire surface of the pillar on the side of cell above the pads is in contact with the cell, Fig. 3.14 shows this in a schematic form.
An effective lamellipodial pressure of 6.7 kPa was calculated. Assuming the force exerted per actin filament to be 6 pN [20] we calculated the number of actin filaments per micron square to be 1117. This value is in agreement with previous studies that have reported this number to be 1370 ± 578 /μm² [19]. Hence the current device also provides a device-independent measurement that can be used to analyze in detail the force-generating mechanisms of the lamellipodium.
3.4 CONCLUSION

In summary, a novel device has been fabricated and developed to measure and map protrusive forces in cells. The unique design of this device enables simultaneous measurement of protrusive and traction forces in cells in an unperturbed state. Of these measurements protrusive forces are critical especially in cancer where it has been reported that in metastasis there is an increase in protrusive force generation [21]. Forces are computed by measuring the displacement of elastomeric microposts using bright field microscopy. The magnitude of protrusive force measured was 1.0 nN and traction force was computed to be 2.7 nN. Furthermore we also estimated the number of actin filaments per micron square which agree with previously reported values thus confirming the accuracy of this method. To the best of our knowledge this is the first demonstration of measuring protrusive forces in fibroblasts in an unperturbed state and simultaneous measurement of traction of protrusive forces on the same device. Future work will be on using the device to measure protrusive forces in different cancer cell lines, this will lead to new insights on cancer biology and development of novel diagnostic tools for cancer research.
References

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Chapter 4

4.1 INTRODUCTION

Silicon of <110> orientation can be wet etched anisotropically with perfectly vertical walls when the mask is aligned so that slow-etching (111) planes form the sidewalls (Fig. 4.1) [1]. Here we used boiling potassium hydroxide (KOH) solution to etch silicon. Grooves and ridges with different heights were fabricated and the substrates had vertical sidewalls and smooth surfaces post KOH etch. Important parameters characterized were, concentration and temperature of KOH and etch time. The substrates were coated with diamondlike carbon by exposure to a fluorocarbon-based plasma, yielding an ultrathin layer of a fluorocarbon material on the surface which has a very low surface energy with excellent antiwear properties. The etched substrates were used as imprint masters and were faithfully replicated and molded in a silicone elastomer. Plasma fluorination can be applied directly to nanoimprint resists as well as to molds used to form elastomer stamps for microcontact printing and other applications requiring easy mold release.

![Etching of <110> silicon. Slow etching (111) planes form vertical sidewalls][2].
4.2 FABRICATION

Silicon wafers pieces (15 X 15 mm) of <110> orientation were subjected to 45 min nanostrip clean, then rinsed with DI water for 5 min, followed by a rinse with acetone and isopropanol, and finally dried with a nitrogen gun. The substrates were spin-coated with 300 nm thick ebeam resist polymethyl-methacrylate (PMMA), molecular weight 495K plus 6% anisole (Microchem) and baked on a hot plate at 180 °C for 60 s to drive residual solvents away from the ebeam resist film. An FEI XL 30 Sirion scanning electron microscope equipped with a Nabity NPGS pattern generator was used for the patterning, with the exposure conditions of 5 keV and a probe current of 10 μA. Resist was developed using a 1:3 solution of methyl isobutyl ketone (MIBK) to isopropanol for 1 min with ultrasonic agitation at 5 °C. The substrate was then rinsed in isopropanol and dried with N₂. To remove any residual resist layer the sample was descummed in oxygen plasma for 30 s. An important thing to note is the orientation of pattern on <110> Si, the patterns has to be written parallel to the edge that has an angle of 70° (Fig 4.2.) this happens because the mask is aligned so that slow-etching (111) planes form the sidewalls.

![Image](image.jpg)

Figure 4.2. Optical micrograph of pattern written parallel to the edge on <110> Si. Arrow indicates the direction of lines
Next, a 25 nm layer of chromium (Cr) followed by a 80 nm layer of gold (Au) was deposited using a Edwards/BOC thermal evaporator. This acts as a mask for KOH etch. Finally, the coated substrate was left overnight in acetone at room temperature for metal lift-off. Following lift-off, samples were rinsed with acetone to remove stray Cr/Au and dried with N₂. Fig. 4.3 (a-e) shows the schematic of the fabrication process.

Figure 4.3. (a-e) Schematic of the fabrication process. (g) Pattern not written parallel to edge results in rough surface with very slow etch rate. (h) Pattern parallel to crystal plane results in smooth vertical side walls but rough bottom surface. Etch conditions 35 % KOH, temperature – 120 °C, time – 2 min. Image in dashed red square shows a zoomed in view.
The patterned substrates were wet etched using various concentrations of KOH solution stirred on a hot plate at different temperatures and time. We found that a 45% (by weight) concentration of KOH with D - H₂O at 135⁰C for 60 s results in an etch rate of 15 - 20 μm/min. Fig 4.4(a,b) shows the etched substrates at different conditions.

![Etched Substrates](image)

Figure 4.4. Optimum experimental conditions result in vertical sidewalls with ultra smooth surface. Etch conditions - 45% KOH, 140 ⁰C, with different etch times. (a) 20 s (b) 60 s. Image in dashed red square shows a zoomed in view.

Due to different etch rates along <111> and <110> the width of the lines gets reduced as well. In case we started with a width of 1.6 μm and after 60 s etch the width was 400 nm, thus the etch rate along <111> plane is 1.2 μm/min. An important point note is a difference of an order of magnitude in the etch rate along <110> and <111> plane.
Figure 4.5. Water and glycerol contact angles as a function of rf discharge power, for different values of the chamber pressure after plasma fluorination using (a) CHF$_3$ and (b) C$_4$F$_8$ [3].

4.3 RESULTS

The fabricated substrates were imprinted in resist and molded into PDMS. Imprinted PMMA film was subjected to plasma fluorination prior to the PDMS application. While the removal of PDMS from the mold is sometimes difficult, in case of fluorinated PMMA mold the separation was easy, and high-aspect-ratio (1:10) PDMS structures came out clean and free of defects. Fig. 4.5 shows the contact angles of water and glycerol after plasma fluorination using CHF$_3$ and C$_4$F$_8$ as a function of rf discharge power, for different values of chamber pressure.
4.4 CONCLUSION

We report the etching of Si (110) using KOH solution at high temperature. This can be used as an alternative tool for fabricating silicon microstructures with vertical sidewalls and smooth surface. It was found that a 45% (by weight) concentration of KOH with D - H$_2$O at 135°C for 60 s results in an etch rate of 15 - 20 μm/min. A smooth Si surface after etching and a constant etch rate leads to precise and reproducible production.
REFERENCES


Chapter 5

5.1 INTRODUCTION

The role of plasma membrane (PM) area as a critical factor during cell motility is poorly understood, mainly due to an inability to precisely follow PM area dynamics. To address this fundamental question, we developed static and dynamic assays to follow PM area changes during fibroblast spreading. Because the PM area cannot increase by stretch, spreading proceeds by the flattening of membrane folds and/or by the addition of new membrane. Reducing PM area reduced spread area, and, in a reciprocal manner, reducing spreadable area reduced PM area, indicating the interconnection between these two parameters.

5.2 EXPERIMENTAL

5.2.1 Fabrication

Silicon wafers pieces (15 X 15 mm) were subjected to 45 min nanostrip clean, then rinsed with DI water for 5 min, followed by a rinse with acetone and isopropanol, and finally dried with a nitrogen gun. The substrates were spin - coated with 300 nm thick ebeam resist polymethyl-methacrylate (PMMA), molecular weight 495K plus 6% anisole (Microchem) and baked on a hot plate at 180 °C for 60 s to drive residual solvents away from the ebeam resist film. An FEI XL 30 Sirion scanning electron microscope equipped with a Nabit NPGS pattern generator was used for the patterning, with the exposure conditions of 5 keV and a
probe current of 10 μA. The master was designed with four rectangular regions of circles
with different diameters (3, 5, 10, 20, 30, 40, and 50 μm).

Resist was developed using a 1:3 solution of methyl isobutyl ketone (MIBK) to
isopropanol for 1 min with ultrasonic agitation at 5 °C. The substrate was then rinsed in
isopropanol and dried with N₂. To remove any residual resist layer the sample was
descummed in oxygen plasma for 30 s. Next, a 50 nm layer of chromium (Cr) was deposited
using Edwards/BOC thermal evaporator. This acts as a mask for Si etch. For lift off the
substrate was left in acetone at room temperature for 20 min. Following lift-off, samples
were rinsed with acetone to remove stray Cr and dried with N₂. Fig. 5.1 shows the schematic
of the fabrication process. The patterned masters were then reactive ion etched to a height
of 4.2 μm in a mixture of C₄F₈: SF₆ (50:1) for 20 minutes at an ICP power - 300 W and rf
power – 50 W and a pressure of 20 mT using an Oxford ICP etch tool. Finally the chrome
was stripped by dipping the samples in CR-100 Chromium Etchant (CYANTEK
CORPORATION) for 5 min.
Chapter 5. *Microcontact Printed Substrates for Understanding Plasma Membrane Area Dynamics*

Figure 5.1. (a-h) Schematic of the fabrication process for Si master.

Figure 5.2. (a) Schematic thermal imprint, resist on glass is imprinted using a Si master. (b) Etched Si master. (c) Imprinted resist on glass. (d) Molded PDMS.
The Si master obtained above was cleaned in piranha for 2 hours at room temperature followed by a 60 s O₂ plasma clean. Then the substrate was subjected to an antiadhesion treatment, this was done by silanizing the substrate in vacuum with vapor phase Tridecafluoro-1,1,2,2-Tetrahydrooctyl-1-Trichlorosilane (United Chemical Technologies) for 12 hours. This substrate (Fig. 5.2b) acted as a master for nanoimprint lithography (NIL). NIL was done using a Nanonex BX-200 nanoimprint system, with polymethyl-methacrylate (PMMA) (Mw = 950 K) as the imprint resist. The PMMA thickness was 5 μm. Typically, the imprint was performed at 3.45 MPa, and 200 ° C for 6 min (Fig. 5.2c). Imprinted resist was treated with a vapor-phase fluorosilane to facilitate removal of the polydimethylsiloxane (PDMS) stamp. PDMS (Sylgard 184, Dow Corning) was mixed with thoroughly with its curing agent in a 10:1 ratio and degassed in a vacuum chamber for 10 min to remove the trapped air. It was poured over the imprinted resist and cured at 65 ° C for 12 h to obtain the stamp for micro contact printing (Fig. 5.2d).

Figure 5.3. Schematic of defects that interfere with clean micro contact printing [1].
An important thing to note is that the design of Si master is crucial for successful micro contact printing. The softness of an elastomer limits the aspect ratio of microstructures in PDMS. When the aspect ratio (diameter/height) is too high or too low, the elastomeric character of PDMS will cause the microstructures in PDMS to deform or distort and generate defects like sagging or pairing in the pattern (Fig. 5.3). The sagging of PDMS caused by compressive forces between the stamp and the substrate excludes the use of microcontact printing for patterns with widely separated (d\geq 20h) features [1].

Figure 5.4. Schematic of microcontact printing [http://gmwgroup.harvard.edu/index.php].

### 5.2.2 Microcontact printing of pattern

Glass coverslips no. 1.5 (Corning Life Sciences, Acton, MA) were cleaned for 1 h in a concentrated 70% H$_2$SO$_4$ : 30% H$_2$O$_2$ ("piranha") solution, rinsed thoroughly with deionized water, and dried by rinsing in acetone and baking at 130°C for 10 min. Then, 2.5 nm of a 60/40 silver-palladium alloy was deposited on a 1.5-nm Ti adhesion layer via e-beam
evaporation using a Semicore SC2000 evaporation system immediately after cleaning. For each substrate, the PDMS stamp was dipped in a 2 mM ethanolic solution of HS-C18 thiol, dried with argon gas, and placed in contact with the substrate for 1 min, after which the substrate was soaked for 2 h in 2 mM ethanolic solution of HS-C11 EG3 thiol to render the nonstamped areas nonadhesive (Fig. 4). Samples were rinsed with ethanol, dried with argon, and stored at 4°C until the fibronectin coating (Fig. 5.5). One to 5 d before an experiment, substrates were incubated for 1.5 h with 10 μg/ml full-length FN (Roche Diagnostics) at 37°C and rinsed with PBS. Substrates are kept at 4°C until use.

Figure 5.5. Microcontact printing of patterned circles coated with fibronectin (FN) onto which cells have been spread for 45 min. The pictures present a reconstruction of several images taken of the same pattern in order to present the half part of a patterned region. The left reconstructed image presents the specific coating of the circles with alexa-546 coupled fibronectin.
5.2.3 Plasma Membrane Area Measurement

N-(3-triethylaminoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide (FM1-43) or 1,1’-didodecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (DiIC12) were prepared following the manufacturer’s recommendations (Invitrogen). Cells were spread on fibronectin precoated coverslips (10 µg/ml; 2 h in PBS at 37°C for total internal reflection fluorescence microscopy [TIR-FM] recording of DiIC12 uptake and epifluorescence recording of FM1-43 uptake) or fibronectin-coated 35-mm plastic dishes (25 µg/ml; 2 h in PBS at 37°C, for epifluorescence recording of DiIC12 uptake). Cells were washed three times with ice-cooled Ringer’s solution and incubated for 25 min on ice in Ringer-DiIC12 (2 µM) or Ringer-FM1-43 (4 µM). For FM1-43 uptake, cells were rapidly transferred to an IX81 inverted microscope (Olympus Imaging America, Center Valley, PA). DIC and epifluorescence images were captured for <5 min at 0°C using a 20 X objective (UPlan Apo 20 X 0.80 numerical aperture [NA] oil). A Roper Scientific CoolSNAP fx cooled charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) recorded 16-bit digital grayscale images from the microscope. For DiC12 uptake, after three washes with ice-cooled Ringer’s solution, cells were analyzed with the same microscope used for the FM1-43 before spreading assay. A mercury burner associated with a 488-nm excitation filter was used to record the epifluorescence intensity. Texas Red-coupled wheat germ agglutinin (WGA) (4 µg/ml in Ringer’s solution) was prepared following the manufacturer’s recommendations (Sigma-Aldrich), and the dye was used following the same protocol developed for the DiIC12.
Figure 5.6. (a) Example of patterned circles coated with fibronectin (FN) onto which cells has been spread for 45 min and incubated at 4°C with FM1-43. (b) FM1-43 fluorescence intensity (representing total PM area) was plotted as a function of substrate contact area for one typical experiment. Each black dot represents a cell. Mean fluorescence intensity, red dashes; best fit for mean fluorescence intensity, blue line. For unlimited substrate contact area, the plotted area represents the average of substrate contact areas on that region.

5.3 RESULT

Substrate contact area affects PM area

If the substrate contact area was related to PM area, limiting matrix contact area could limit PM area. Microcontact printing [2] was used to pattern fibronectin-coated circles of different areas onto a nonadhesive background (Fig. 5.6a). We found that PM area increased with the substrate contact area above a threshold of 706 \( \mu m^2 \) (Fig. 5.6b). Moreover, the PM area increased by \( 53 \pm 6\% \) (Fig. 5.6b; SEM; \( n = 2 \) independent experiments; 45 min of spreading) for cells that spread on continuously coated fibronectin regions, compared with
cells spread on 30-µm-diameter circles (706 µm²; Fig. 5.6b). This value was similar to that observed for spreading from 5 to 30 min (i.e., a 41.6% increase in PM area). Thus, the increase in PM area for fully spread cells is related to final substrate contact area when cells spread over ~1000 µm².

5.4 CONCLUSION

The results of this study provide the direct observation and proof of what really appendes in the cells and how plasma membrane area is modulated in vivo. This is an important step forward in the field showing that more than any change in protein or lipid composition, a simple change in the total plasma membrane area could lead to modifications in cell behavior. In conclusion, the plasma membrane area is a critical biophysical parameter during cell shape changes.
REFERENCES
