ENGINEERING CELLULAR MICROENVIRONMENTS FOR DIRECTED CELL GROWTH AND MIGRATION

BY
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DISsertation
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Abstract

Microscale systems have been finding increasing and important applications over the recent years in tissue engineering and biological studies. Recent advances in microfabrication technologies have enabled these systems to precisely control the cellular microenvironments and investigate cell behavior in vitro. This work aims at integration of advances in life sciences and microfabrication techniques to address fundamental biological questions underlying cellular behavior. We specifically focus on two applications related to human health (1) using micropatterned surfaces for tendon regeneration, and (2) using microfluidic platforms for neutrophil chemotaxis. Both of these applications are of crucial importance in modern biological and medical advancement. We have developed micropatterned platforms for systematic analysis of tenocyte behavior on topographical surfaces. These studies investigate the role of microtopographical features on the cytomorphology, alignment, proliferation, extracellular matrix synthesis, and gene expression of tenocytes. We have also demonstrated microfluidic platforms as powerful tools for quantitative analysis of intracellular and extracellular events in neutrophil chemotaxis. Using fluorescence-labeled cells, these microfluidic platforms are used to investigate the localization of key regulatory molecules, Actin and PHAKT involved in signaling pathways during chemotaxis. Furthermore, we employ these platforms to understand macroscale neutrophil response to multiple chemoattractant gradients. The analysis of cell migration behavior in response to single and opposing gradients of chemoattractants have begun to elucidate how neutrophils integrate and prioritize multiple chemotactic cues. These results provide insight into complex cellular behavior and involved mechanisms and should aid in the design of novel therapeutic strategies to treat human health disorders.
To

my genuine well-wishers
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Chapter 1 Introduction

Several factors influence cell behavior *in vivo* and determine characteristic cell response to stimuli. These factors include interactions with other cells, interactions with the extracellular matrix, and systemic factors. Current research aims at replicating *in vivo* cellular microenvironments to understand the individual and coordinated role of such factors. Microscale platforms provide unique functionalities of controlling the cellular microenvironments in a highly precise manner, thereby enabling us to study aspects of cell biology not possible with conventional, macroscale platforms.

1.1. Microfabrication and cell studies

Cellular micronenvironment in a native tissue comprises primarily of a complex blend of extracellular matrix molecules, soluble factors and numerous cells of various types [1]. Collectively, these components present a complex array of inputs to cells. Cells undergo a series of signaling pathways processing these inputs and elicit a peculiar response.

The various factors that influence cell behavior *in vivo* can be classified broadly as (i) interactions with other cells, (ii) interactions with the extracellular matrix, and (iii) systemic factors. Cell-cell interactions, comprising of diffusible signaling and juxtacrine signaling are shown to be crucial in numerous biological processes, such as tumor growth [2], atherosclerotic plaque formation [3], and stem cell differentiation and self-renewal [4]. The microenvironments in which cells are embedded control various signal transduction processes that direct cell survival, cell cycle progression, and the expression of different phenotypes, along with providing mechanical stability [5].
Systemic or local inflammation plays an important role in determining immune behavior of cells [6]. The levels of complexity involved in cellular interactions necessitate development and application of novel approaches in order to gain a better understanding of the intricate cellular mechanisms and behavior.

In the recent years, there has been increasing use of microfabrication techniques in biological studies [7,8] owing to its various merits. Advancement in microfabrication methods allow to work at small scales suited perfectly for biological studies, perform multiple assays on a single device and integrate different processes on a single chip. These specific advantages make them specifically suitable for meeting demands of commercially high-throughput, low-volume consumption technologies such as whole-genome sequencing projects, and combinatorial approaches to drug discovery [9].

Various kinds of platforms for biological assays [10], bioreactors [11], drug delivery devices [12], biosensors [13,14] have been successfully created and utilized to gain an insight into cell biology. A microwell array has been demonstrated as a controlled way of analyzing and sorting individual cells for dynamic culture and clone formation (Figure 1.1) [15]. The microwell arrays have been used in several biological investigations such as analysis of leukemia cancer cells with respect to their clonogenic properties [16], maintaining pluripotency of stem cells [17], protein binding assays [18], gene amplification and mutation analysis in individual wells [19]. Powers et al. [21] have demonstrated design, fabrication and performance of bioreactors that enable both morphogenesis of 3D tissue structures under constant perfusion and repeated in situ imaging of tissue structure and function using two-photon microscopy. Mass transport
in such 3D cultures can be significantly enhanced using micro-capillaries in bioreactors [22].

Figure 1.1 Microwell array device with integrated microfluidic components for enhanced single cell analysis [20] (Reproduced with permission).

Advances in micro- and nanotechnologies have led to the development of novel drug delivery microdevices or components of therapeutic microdevices that can improve therapeutic benefits of drugs, such as microneedles (Figure 1.2), and implantable drug delivery microdevices (Figure 1.3). Microneedles offer great advantages in targeting drug delivery to a specific region or tissue in the body, thus avoiding detrimental effects that can result from administering certain drugs systemically. The precise shape in combination with structural robustness allows them to be a viable option for painless transdermal delivery [23]. Also, the implantable microdevices have been shown to be of
great potential in terms of their capability of very accurate dosing, complex release patterns, local delivery and biological drug stability enhancement [24,25].

Figure 1.2 Microneedles for painless transdermal drug delivery. For comparison, a conventional hypodermic needle is also shown in panel D [23] (Copyright (2003) National Academy of Sciences, U.S.A).

Figure 1.3 Active-delivery devices for rapid, controlled delivery. (a) Schematic of device showing actuation mechanism. (b) In vitro release of methylene blue from an implantable rapid-drug-delivery device [24] (Reproduced with permission).

Efficacious cell patterning has been demonstrated by passively patterning them by random seeding on surfaces modified with cytophilic and cytophobic regions, and more recently by active deposition on the surfaces via optical or electrical forces or even directly printing them on substrates (Figure 1.4) [26,27].
1.2. Microfluidics and cell studies

Microfluidic systems have emerged to be of great potential in various biological applications including bimolecular separations [28], enzymatic assays [29], polymerase chain reactions [30] and chemotaxis assays [31]. Microfluidic platforms provide immense advantage over conventional, macroscopic systems while consuming much smaller amounts and solvents [32]. In addition, the microscale dimensions enable us to access phenomena that are not easily accessible on the macroscale. The laminar,
turbulence-free flow with precise control over flow properties enables the extraction of high-quality data from cell based assays. Most of the cell-based microsystem research is aimed at creating 'lab-on-chip' microsystems incorporating several steps of an assay into a single system [8,33]. Integrated microfluidic devices have potential to perform precise, rapid and reproducible measurements on small reagent volumes, thereby overcoming the limitations of painstaking labor-intensive and error-prone laboratory manipulations (Figure 1.5).

Developments in microfluidics can be greatly attributed to recent progress in material science, along with the significant contribution of microfabrication technologies from the microelectronic industry. Microfluidic devices can be fabricated in a large selection of thermoplastic or elastomeric materials possessing a wide range of chemical, mechanical and optical properties, tailored for specific applications [34]. Furthermore, the surface of the materials can be modified by physical and chemical methods, allowing access to an almost unlimited variety of surface properties.
Figure 1.5 A collection of microsystems enabling cell-based assays, covering all the steps from cell culture, through selection and treatment, to biochemical analysis [8] (Reproduced with permission).

Microfluidic platforms have been successfully demonstrated for cell cultivation, growth and differentiation in various types of mammalian cells [35]. Huang et al. [36] has developed a device for the side-by-side patterning of multiple gel types that allows the cultivation of metastatic breast cancer cells next to macrophage cells and real time imaging of the interaction of cells to both autocrine and paracrine signaling (Figure 1.6).
Lii and co-workers [37] developed a 3D microenvironment for real-time monitoring of an array of mammalian cells. Yang et al.[38] presented a multilayered device, manufactured from PDMS, for the separation of chemical stimulants over single living cells vertically through aqueous-phase separation by the use of laminar flow.

Figure 1.6 Microfluidic device with spatially patterned gels for three-dimensional multicellular co-cultures [36] (Reproduced by permission of The Royal Society of Chemistry).

Droplet-based microfluidics has attracted a lot of interest recently due to its wide applications to chemical and biological assays [39,40]. Droplets have been applied to investigate or process cells with high throughput. Cells encapsulated in microfluidic droplets were used to study gene expression [41,42], to examine effect of reagents on cells [43-46], and to process cells for tissue engineering [47,48]. Significant effort and advancement has been made for single cell encapsulation and cell sorting using microfluidic droplets [49-51].
The use of fluidic properties at microscale allows focusing, guiding and patterning of cells. This can be accomplished by several phenomena such as microvortex [52], surface patterning of receptors [53], standing acoustic waves [54] and hydrophoretic focusing [55]. Microfluidic devices have been demonstrated for transport [56] and precise positioning of cells [57]. Sequential trapping, labeling and content extraction of individual cells opens the possibility to study individual cell lysis [58]. Similarly, it is possible to trap and properly pair different types of cells as fibroblasts, mouse embryonic cells and myeloma cells (Figure 1.7) [59].

Figure 1.7 Microfluidic device for cell capture and loading [59] (Reproduced with permission).
High-throughput microfluidic devices have been demonstrated for precise cell counting and flow cytometry. Rosenbluth and co-workers [60] measured single cell transit times of blood cell populations passing through in vitro capillary networks. Golden et al. [61] developed a multiwavelength microflow cytometer using grooved microfluidic channel generated sheath flow. Holmes et al. [62] demonstrated a cytometer device based on single cell impedance measurements. Fu et al. [63] used hydrodynamic focusing to concentrate particles in the center stream of cytometer device. Cells or particles are detected and counted by laser induced fluorescence. Micro flow cytometry devices have also been demonstrated using optical fluorescence detection with resistive pulse sensing enhanced by metal oxide semiconductor field effect transistor (MOSFET) [64], chemiluminescence detection [65], and immunoaffinity-based capture [66].

Moreover, the high precision and control over flow properties make the microfluidic devices a viable option for separation, sorting and trapping of cells. In a recent study, separation of amniotic fluid mesenchymal stem cells was demonstrated with a device utilizing louver-array structures [67]. Wu et al. [68] employed specific channel geometry in their device that resulted in a soft inertial force effect on the cells and separated bacteria from human blood cells based on size differences. In another variation, asymmetric channel geometry was used for differential inertial focusing for cell separation in blood [69]. Separation and sorting of different types of cells based on their difference in the dielectric properties has been effectively demonstrated in various biological applications [70-73]. The principles of acoustic waves [74], optofluidics [75], and magnetic forces [76] have been used to perform on-chip cell sorting.
Lab-on-chip microfluidic devices find increasingly useful applications in clinical diagnostics [77], cancer research [78], stem cell research [79], drug discovery and screening [80], neuroscience [81], microbiology [82], cell mechanics [83], intra and intercellular signaling [84], and tissue engineering [85].

1.3. Motivation

A substantial amount of current biological research is aimed at understanding the fundamental mechanism of intracellular, intercellular and cell-extra cellular matrix interactions. Understanding cellular mechanisms is the foundation of addressing health related problems like tendon and ligament repair [86], inflammation, arthritis, and others [87]. The motivation of these studies is to employ recent advances in microfabrication and microfluidic methods to develop powerful tools to gain an insight into cellular behavior and cellular mechanisms. The improved understanding of cellular mechanisms will provide rational basis for guiding tissue formation and aid in development of new therapeutic treatment strategies.

1.4. References


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Chapter 2 Topographical control of cell behavior: literature review

2.1. Introduction

Last few decades have seen an unprecedented increase in number of injuries and emerging diseases all over the globe. This rise can be attributed to changing lifestyles, environmental degradation, and several other factors and is driving the medical research to explore solutions to these problems. All fields of medicine have experienced an increase in number of surgeries, number of implants, and the number of transplants. However, all of these have their limitations. Epidemiological research shows that many of the implants fail because of rejection by body and may even result in conditions dangerous for the life of the patient. We still do not understand well enough the compatibility of implanted device with host body (humans or animals).

The enormous mystery behind nature is too hard for an individual to unravel on one's own. In recent years, a lot of interdisciplinary research has been done involving various fields of engineering, and life sciences to understand the intricacies of nature. One of the most important factors in body response to implanted device is the initial reaction of cells towards the device. Another factor is that we still do not understand the fundamentals behind innumerable mechanisms going on within the cell. So, researchers are trying to understand the intracellular, intercellular and cell-substrate interactions. By gaining a better understanding of all these, we hope to be able to develop better clinical strategies.

Tissue engineering is one such branch of study that aims at regeneration of tissues. Surgeries and implants being limited in approach have led to emergence of tissue
engineering that incorporates insights and expertise from various disciplines. By controlling the factors that can facilitate tissue repair and regeneration, it can be used to tackle the issues never understood before.

Understanding cell-cell and cell-substrate interactions is the foundation of tissue repair. A lot of research is aimed at improving the fundamental mechanism of cell-cell and cell-substrate interactions. Understanding the nature of these interactions and mimicking it is likely to help us to create the native cellular environment on lab scale and help in tissue regeneration.

With advanced microfabrication tools, manipulation of chemical nature of surface and patterning of several kinds of topographical features has been made possible [1-4]. The novel patterning methods enable us to mimic the various topographical and chemical cues that exist naturally in the body.

2.2. Phenomenon of contact guidance and its relevance in tissue regeneration

Various cells grow in aligned manner in response to substrate features, physical, chemical or combination of both, the phenomenon known as contact guidance [5-7]. Such aligned growth of cells in turn facilitates normal regrowth and functioning of tissue [8-10]. Various research groups have investigated the role of contact guidance in peripheral nerve repair [11], regeneration of ligament [12] and production of tendon substitutes [13], and control of microvascular repair [14].

Extensive research has been done to study cell behavior in response to variation in substrate properties [15-19] particularly with respect to cellular alignment and growth [20-22].
Several excellent reviews have been published covering various aspects of cell contact guidance on micropatterned surfaces [23-30][()]. A few specific reviews have also been published dealing with specific type of cells namely axon cells [31] and endothelial cells [32].

In this chapter, we present an overview of current developments in the topographical patterning approaches for controlling cell behavior.

2.3. Reasons behind contact guidance

The mechanism behind contact guidance has not been yet clearly understood. Several questions have been raised and investigated by researchers regarding cell response to topographical cues. Is the entire cell aligned or only the cytoskeleton? Which cellular components are aligned? Is alignment of nucleus also related to alignment of cell? Which components align first and do they control alignment of other components over time?

Experiments done with fibroblasts on grooved substrates have shown the alignment of the following components in various cases: entire cells; actin microfilaments; microtubules; focal contacts, extracellular matrix proteins [25,33]. Oakley et al. [34] reported that microtubules were the first cellular component to align along the grooves (20 mins after seeding), followed by other components actin filaments, focal contacts and finally the entire cell [35]. Wojciak-Stothard et al. [36] observed acting condensation and vinculin staining along the pattern boundaries 5 min after seeding. The alignment of entire cell has been observed even before a well-organized actin cytoskeleton has been developed [33]. Cells have also been observed to align either with an intact actin microfilament system or with an intact microtubule system [33]. All
these observations illustrate the complexities behind the contact guidance phenomena. Braber et al. [37] observed from their work on rat dermal fibroblasts that orientation of both intercellular and extracellular proteins is influenced by topography of surface.

Several hypotheses have been suggested to explain the phenomena of contact guidance. One of the hypotheses proposes that the stiffness of focal adhesion contacts is responsible for contact guidance [38]. The focal contacts become aligned along the groove direction and polarize the actin cytoskeleton originating from these contacts. Since the motility forces can only be applied along the long axis of rectangular focal contacts, they cause entire cells to align.

In a recent study with endothelial cells on silicone surfaces [39], alignment of F-actin and focal adhesions was observed in case of aligned cells (Figure 2.1). The “mechanical restriction” imposed by substrate topography lead to the formation of linear bundles of F-actin. The inherent stiffness of F-actin was supposed to be responsible for cell alignment on underlying topographical surfaces.

![Figure 2.1 F-actin staining (orange) and corresponding vinculin staining (green) at focal adhesions in Endothelial cells on (a), (b) smooth, and (c), (d) 5 µm PDMS. Scale bar: 20 µm. [39] (Reproduced with permission).]
Similar alignment of F-actin was observed in Vascular Smooth muscle cells (Figure 2.2) on Fibronectin coated PDMS substrates [40].

Figure 2.2 F-actin staining (red) in smooth muscle cells on grooves of widths (A) 19, (B) 48, (C) 79 µm, and (D) smooth surface (Sarkar, 2005). Alignment is along the long axis of the cell (indicated by white arrows). (1) and (2) denotes grooves. Scale bar: 12 µm. [40] (Reproduced with permission).

Focal adhesions have also been shown to play a key role in cell spreading, migration and division [41]. Interestingly, Teixeira et al. [42] found only few focal adhesions in their studies and cell alignment was observed even in absence of alignment of focal adhesions and stress fibers [5]. Thus, it was inferred that cellular alignment was not always contingent upon the presence of focal adhesions. Still, the switching of cellular alignment from parallel to perpendicular direction to the topographical features was accompanied by changes in focal adhesion structures [42].

Charest et al. [43] also made a similar observation of lack of focal contacts due to lack of adhesive area and absence of their alignment with features (Figure 2.3). These observations corroborate a more tactile mechanism of guidance with filopodia or lamellipodia. Cells perceived the surface with these cytoplasmic projections. At point of contact with the substrate, these were bent and subsequently aligned with the grooves
and ridges, thereby minimizing the generation of forces perpendicular to walls. Thus their propagation is hindered by the wall and is directed along the pattern direction.

![Figure 2.3](image)

**Figure 2.3** (A) Perpendicularly aligned cell on 70 nm wide ridges on a 400 nm pitch. (B) Detail of previous cell. Filopodia were aligned perpendicularly to the patterns. We also observed filopodia aligned along the patterns. (C) Parallel aligned cell on 1900 nm ridges on a 4000 nm pitch. (D) Filopodia were guided by the topographic pattern [42] (Reproduced with permission).

Microspikes, the actin-rich extensions in lamellipodia, have also been found to play a key role in formation of focal adhesions via actin polymerization. Microspikes probe the environment and on finding a surface suitable for attachment, formation of focal adhesions and mature actin fibers take place. Thereafter, actin filament formation and elongation along the direction of patterns leads the cell to equilibrium aligned state (Figure 2.4) [44].
Another hypothesis proposes the alignment of cells occur so that cells reach biomechanical equilibrium with the net resultant forces minimized, favorable for their differentiation (Figure 2.5). According to the tensegrity models, the anisotropic geometry of the patterned surface establishes stress and shear free planes that cause cytoskeletal components to undergo reorganization [34,35].

Walboomers et al. [25] proposed that the microgrooves create a pattern of mechanical stress thus effecting cell spreading and alignment. The reaction to forces generated on actin is the underlying mechanism that causes contact guidance. Cells protrude in all directions and explore their environment. The propagation of these structures is directed by extension of actin filaments (Figure 2.6). When an extension faces a hindrance in form of a surface ridge, it can propagate only along the direction of
groove and cell spreading is allowed only along that direction. This hypothesis is substantiated by studies that have detailed images of leading edge of migrating cells.

![Figure 2.6 A schematic showing formation of a new focal adhesion](image)

Micropatterned surfaces have also been shown to induce extracellular matrix reorganization which influences directional cell response [40]. In a study with vascular smooth muscle cells on Fibronectin-coated PDMS grooved patterns, cell-mediated remodeling of Fibronectin was observed (Figure 2.7). The fibrillar structure of Fibronectin was due to both cell-derived and pre-adsorbed Fibronectin and showed strong dependency in its occurrence and orientation on the feature dimensions. These regions can serve as sites for cell-generated contractile forces for propagation of cells throughout the ECM.
Figure 2.7 Cell-mediated remodeling and deposition of Fibronectin. Groove widths are (A),(E) 19 µm, and (B),(F) 48 µm, (C),(G) 79 µm, and (D),(H) unpatterned. Fibronectin fibrils are indicated by white arrow. Fibrillar FN in (F) and (G) observed in areas not covered by cells (B), (C), indicating cell-mediated remodeling and deposition of FN (black arrows). FN staining (G), (H) yields less fluorescent intensity within the cells (red block arrows). Scale bar: 12 µm. [40] (Reproduced with permission).

The preferential adsorption of ECM proteins to the discontinuities present on grooved surfaces has also been hypothesized to be responsible for alignment of focal contacts and contact guidance [38]. Microtextured surfaces can produce local differences in surface-free energy that can in turn affect deposition pattern of substratum-bound attachment proteins. Also the spatial arrangement and conformation of proteins is determined by substratum surface properties [30,46,47]. Curtis et al. [48] suggests that the cells may be reacting directly to the topography or to some derivative
feature, such as the absorption of specific proteins that aid or hinder cell adhesion. The topographically determined distribution and conformation of adsorbed proteins on different areas of the substrate in turn promote preferential integrin-mediated adhesion of cells [37].

Considering the variation in cell types, substrate types and range of cell-substrate interactions, it still remains a challenge to come to a unified theory that explains the phenomena of contact guidance.

2.4. Microfabrication of patterned surfaces

Recent advancements in fabrication methods have enabled us to create patterned surfaces with micron to nanosize features. The ranges of substrates that can be patterned vary from conventional microelectronic substrates to novel biodegradable polymeric substrates.

The first generation patterned surfaces were fabricated primarily on inorganic substrates like silicon [49]. The necessity for in vivo studies and implantation has lead to extensive research and development in patterning of biodegradable polymer substrates. Compression molding and solvent-casting have been used to transfer micropatterns from quartz and silicon dies onto biodegradable PDLA films [50] and the degradability was found to differ depending on the process of fabrication. Papenburg et al. [7] fabricated highly porous micropatterned scaffold sheets to incorporate the merits of porous features for viability with respect to nutrient transport with those of micropatterns for directing cell growth. The 2-D scaffold sheets fabricated using phase separation micromolding can be stacked up to form multi-layer 3-D scaffolds. Vernon et al. [51] made a breakthrough by fabricating a self-supporting, micropatterned scaffold
entirely from a native, biological substrate, fibrillar collagen I. Topographical features approaching sub-10 nm length scale have been patterned at the surface of thermoplastic polymers. Gadegaard et al. [52] used injection molding to mimic the complex nanostructured fibrillar collagen network in various thermoplastic polymers like polypropylene. Collaterally situated planar and nanopillars have been fabricated using colloidal lithography to monitor cell reactions on two surfaces simultaneously [53].

Many biological applications require large area spanning over several centimeters patterned with nanosize features, which is beyond the limitations of conventional patterning methods. Lenhert et al. [46] used Langmuir-Blodgett lithography to fabricate nanopatterned surfaces over several square centimeters on silicon surfaces and transferred them to polystyrene by nanoimprinting. The technique is simple and allows patterning over a wide range of biomaterials.

A wide variety of microfabrication methods have therefore been used to create patterned substrates. The micropatterned surfaces obtained from these methods are shown here (Figure 2.8-Figure 2.17).
Figure 2.9 Phase inversion method. SEM images of polyurethane membranes (a) Top surface, and (b) Cross section [15] (Reproduced with permission).

Figure 2.10 Phase separation micromolding (PSμM). SEM images of 5% PLLA films obtained by PSμM with non-solvent at 4 °C [7] (Reproduced with permission).

Figure 2.11 3-D scaffolds obtained through (a) clamping two films, and (b) rolling up one film [7] (Reproduced with permission).
Figure 2.12 Grooved collagen membranes replicated from plane-ruled diffraction gratings. 30 grooves per mm. Major and minor faces of the grooves (asterisks, arrows) and free edges (arrowheads) are indicated. Scale bar: 25 µm. [51] (Reproduced with permission).

Figure 2.13 Grooved collagen membranes replicated from plane-ruled diffraction gratings. 150 grooves per mm. Double-headed arrows indicate groove axes and arrowheads indicate free edges [51] (Reproduced with permission).

Figure 2.14 AFM phase micrographs of (A) a nickel die, and (B) and an injection molded poly(propylene) replica of collagen network. Scale bar: 500 nm. [52] (Reproduced with permission).
Figure 2.15 SEM image showing nanopillars on quartz substrate [53] (Reproduced with permission).

Figure 2.16 Optical micrograph of a topographically patterned polystyrene substrate with a periodicity of 300 nm. Scale bar: 2 µm. Patterns were created on silicon using Langmuir-Blodgett lithography and subsequently nanoimprinted on polystyrene [54] (Reproduced with permission).

Discontinuous edges have also been fabricated to investigate “gap guidance” in cells.

Figure 2.17 SEM images of discontinuous edged surfaces used to study “gap guidance” [55] (Reproduced with permission).
2.5. Directed growth of cells

2.5.1. Range of cells and substrate materials

Table 2.1 Materials used in fabrication of topographically patterned substrates for various cell types

<table>
<thead>
<tr>
<th>Cells</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>Silicone ([37,44,47], PMMA [56], Silicon ([34,57,58], Araldite [59], Epoxy [60], Fused quartz[61], Perspex [62], Poly (3-(tert-butoxycarbonyl)-N-vinyl-2-pyrrolidone)[10], NOA61 polyurethane [63], Quartz <a href="12">64</a>, Polystyrene [44,65], PCL, PCL-collagen nanofibers [66], fibronectin [67], fused silica [36], PLGA [68], Silica[48]</td>
</tr>
<tr>
<td>Schwann cells</td>
<td>PLGA [69], PDLA [50], PCL, PCL-collagen nanofibers[66]</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>Epoxy [60], Ti coated silicon ([70,71], NOA61 polyurethane [63], Silicon [72], SiO₂ [5,42], silicon, Polyurethane [73]</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Poly (glycerol–sebacate) (PGS) [74], silica [48], poly(L-lactic acid)-co-poly(epsilon-caprolactone)[75], PLA [7], PCL [7,48]</td>
</tr>
<tr>
<td>Smooth muscle cells and</td>
<td>Polystyrene[78], PDMS [79,80], PMMA <a href="34">80</a>, polyurethane diacrylate [81], modified PDMS [82], fibrillar collagen membranes [51], Polycarbonate [43], PLA [7], PCL [7], polyesterurethane [83], Quartz [84]</td>
</tr>
<tr>
<td>myoblasts</td>
<td></td>
</tr>
<tr>
<td>Oligodentrocytes</td>
<td>Quartz [85]</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Polystyrene [86,87], PDLA [88,89]</td>
</tr>
<tr>
<td>Neurites</td>
<td>PDLA [50], poly-L-lactate [11], PDMS [90], PCL [91]</td>
</tr>
<tr>
<td>Tenocytes</td>
<td>PGA fibers [92], Polydioxanone (PDS) [13]</td>
</tr>
</tbody>
</table>
2.5.2. Types of topographies studied

Table 2.2 Types of topographical features used for studying cell behavior

<table>
<thead>
<tr>
<th>Topography</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Grooves and ridges</td>
<td>[43,91,93]</td>
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<td>V-shaped grooves</td>
<td>[94]</td>
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<tr>
<td>Steps</td>
<td>[62]</td>
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<tr>
<td>Waves</td>
<td>[74]</td>
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<tr>
<td>Wells and nodes, holes</td>
<td>[43]</td>
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<tr>
<td>Pits</td>
<td>Tapered pits [95-97]</td>
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<td>Inverted pyramids [95,96]</td>
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<td>Pores</td>
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<td>Spheres</td>
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<td>Particle monolayers</td>
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<td>Microfibrils</td>
<td>[51,103]</td>
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<td>Surface roughness</td>
<td>[104]</td>
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2.5.3. Effect of width

Various studies have been conducted to investigate the effect of groove and ridge widths on cell behavior. An important consideration while performing these studies is the typical dimensions of cells so that response of cells to dimensions smaller, equal and larger than that of cells can be determined. The results have been found to vary a lot for different cell types.
Groove width promoted the alignment of Schwann cells with the optimum dimensions of grooves being 10-20 µm, comparable to cellular widths [50]. Brunette et al. [57] investigated the relative orientation of fibroblasts on a smaller set of minor grooves on the floor of the major grooves. Fibroblasts were observed to align both within the grooves and on the ridges. However, cells aligned to major grooves (width 36-162 µm) in preference to minor ones (4-6 µm wide) when both grooves were there simultaneously. The best alignment was observed in case of major groove width (36-78 µm). The studies conducted on rat dermal fibroblasts by den Braber et al. [37] showed that ridge width rather than groove dimensions was the most significant parameter in controlling cell size, shape and orientation. Cells were highly elongated and aligned (within 10 degrees) when the ridge dimensions were smaller than 4 µm. Papenburg et al. [7] studied the alignment of myoblasts in 20 µm wide grooves in comparison to 250 µm wide grooves (Figure 2.18). At higher confluence, cells start to grow over the ridges but maintained their alignment. Cells grown on interconnected channels showed the alignment of cells along the direction of the channels. Oakley et al. [33] observed that alignment of fibroblasts was much more in narrow grooves (1-9 µm pitch) compared to wider grooves (30 µm pitch). In case of 30 µm, the cells were confined within the grooves, and in case of narrower grooves, they bridged over several grooves. Ricci et al. [65] observed profoundly altered morphologies, reduced growth rates, and directional growth in “dot” colonies grown on microgrooved substrates, when compared with colonies grown on flat, control surfaces. Walboomers et al. [44] observed that alignment was established fastest on the narrowest grooves compared to the wider grooves. Wójciak-Stothard et al. [36] found that patterned substrata substantially
activated cell spreading and elongation and significantly increased the persistence and speed of cell movement. Bettinger et al. [74] demonstrated in their studies on micropatterned biodegradable substrates that Bovine aortic endothelial cells cultured on substrates with smaller pitches 2 µm, exhibited a substantially higher frequency of cell alignment and smaller circularity index. Lam et al. [79] studied the alignment of skeletal muscle cells by use of continuous micropatterned wavy silicone surfaces, with features sized 3, 6 and 12 µm in periodicity and found that wave features with 6 µm periodicity produced the most healthy, aligned muscle cells.

![Figure 2.18 Alignment of mouse myoblasts, C2C12 as a function of micropattern designs. Images show cells on PLLA sheets featuring different patterns, after 4 days, seeding density of 25,000 cells/cm². The arrows indicate channel direction. (a, c, e) light microscopy (magnification 10X, methylene blue used for staining), (b, d, f) confocal fluorescence microscopy [7] (Reproduced with permission).](image)

Some studies have found that cell shape is predominantly influenced by substrate features and there is not so significant effect on cell alignment as such. Gray et al. [105] observed cell elongation on decreasing the channel width from 225 µm to 25 µm.
Sarkar et al. [40] found in the studies of vascular smooth muscle cells (VSMCs) on PDMS microtextured scaffolds that alignment did not change significantly on varying groove width from 20-70 µm; however cell shape was significantly influenced by groove dimensions. The aspect ratio on narrow grooves 20-50 µm was observed to be 11.5. At higher cell densities, cells retained their elongation and alignment.

The orientation of ECM proteins has also been observed as a function of topographical feature dimensions. den Braber et al. [37] investigated the relative depositions of bovine and endogenous fibronectin and vitronectin on grooves of dimensions 2, 5 and 10 µm and observed that proteins were significantly more oriented on 2 µm grooved surfaces compared to 5 and 10 µm grooves. Shen et al. [81] observed in their study on A7r5 smooth muscle cells and C2C12 skeletal muscle cells that cells not only grew within the channels but also climbed on the walls and grew on top of the walls (Figure 2.19).

The attachment of cells to ridges or walls rather than grooves usually becomes more pronounced when groove dimensions become narrower. Feinberg et al. [106] investigated the effect of ridge widths on cell alignment and morphology on PDMS
patterned substrates, and also compared them with plasma treated and fibronectin coated patterns (Figure 2.20). 1.5 µm ridge heights in untreated PDMS resulted in significantly greater alignment than in either fibronectin coated or plasma treated substrates.

Figure 2.20 Endothelial cells cultured on smooth and patterned PDMS substrates with different ridge widths. Cells were cultured for 3 days, fixed and then stained with hematoxylin [106] (Reproduced with permission).

The reduction in feature size to nanoscale seems to be a potential parameter to control the cell behavior. Nanogrooves have been demonstrated to limit lateral expansion [93] and promote cell function [77]. However, it would be too early to project
these results for in vivo success considering the enormous complexities involved in in vivo environment.

Cells may have a specific sensing mechanism that enables them to detect optimal topography for their growth and alignment. The possibility has been indicated by a recent study on fibroblast response to PLGA features of different widths. The cell growth was highly stimulated in 150 µm wide channels, whereas smaller and bigger channels showed less than substantial cell growth. These findings are of great relevance in design of substrates for tissue engineering applications [68].

2.5.4. Effect of depth

Groove depth has been shown to be a key factor influencing cell orientation and behavior in many of the studies. However, again the observations vary depending on cell type and cell-substrate interaction.

Andersson et al. [38] investigated the effect of groove depth by varying the groove depth between 40 and 400 nm and keeping the groove and ridge width constant and identical surface chemistry. The alignment of epithelial cells increased with increasing groove depth. Hsu et al. [69] investigated the role of groove depth by varying the depths between 0.5 and 3 µm and found it to be the most important factor in controlling cell response. They concluded 1.5-3 µm to be optimal for cell alignment and growth. Clark et al. [62,107] showed an increase in alignment of cells with increase in step size features on substrates. Lenhert et al. [54] demonstrated significant alignment of osteoblasts on 150 nm deep grooves compared to 50 nm deep grooves fabricated using Langmuir–Blodgett lithography. Walboomers et al. [108] found that the rate of fibroblast orientation was increased by increase of groove depth from 0.5 to 5.4 µm. Uttayarar et
al. [39] cultured bovine aortic endothelial cells (BAECs) on micro to nano grooved PDMS substrates. BAEC alignment, elongation, and projected area were investigated for channel depths of 200 nm, 500 nm, 1 µm, and 5 µm, as well as smooth surfaces (Figure 2.21). The alignment was found to increase with increasing channel depth, with maximum alignment observed at 1 µm depth. The deeper grooves were also found to be more effective in directing locomotion of epithelial cells. Loesberg et al. [109] also concluded depth to be the most essential parameter in controlling cellular alignment. It was observed that given sufficient culturing time, fibroblasts align themselves on groove depths as shallow as 35 nm, provided that ample ridge-surface (150 nm wide ridges) was available to the cells.

![Figure 2.21 Percentage of endothelial cells on smooth and grooved PDMS at 48 hr. The asterisk (*) denotes the significant difference (p < 0.05) between the percentages of aligned cells on grooved surfaces and smooth surfaces. For the grooved surfaces S corresponds to significant difference [39] (Reproduced with permission).](image)

Clark et al. [61] studied the effect of ultrafine nanoscale dimensions 260 nm period grating surfaces (130 nm grooves separated by 130 nm) of various depths (100, 210 and 400 nm) on alignment of various types of cells. MDCK cells showed alignment on topographical patterns and their elongation was depth dependent. In case of BHK cells,
the alignment was a function of depth. However, the outgrowth of neurites from chick embryo neurones, were mainly unaffected by the grating surfaces. Surface topography was also shown to stimulate and govern chondrocyte aggregation, alignment and migration (Figure 2.22).

Figure 2.22 Chondrocytes aggregates formed on flat control silica (A), and on grooves of depth 750 nm (B), 3 micron (C), and 8 micron (D). Cells grow to confluent monolayers on flat surfaces and are contact inhibited with time. On shallower grooves (3 micron), cells also form monolayer aggregates, which tend to be aligned with the direction of the groove long axis. Multilayered aggregates form only on depths of 3 micron or greater [110] (Reproduced with permission).

2.5.5. Simultaneous effect of width and depth

Some of the research studies have clearly demonstrated the effect of both groove/ridge width and depth on cellular alignment and behavior. Clark et al. [107]
showed that alignment had stronger dependence on groove depths and inverse proportion (smaller effect) to repeat spacing i.e. ridge width. The peculiar perpendicular orientation of CNS neuroblasts to topographical substrates was observed only when optimum width 1 micron and depth of features between 0.3 and 0.8 micron on substrate was provided [64,111]. Brunette et al. [57] found that deeper grooves were more effective in directing cell locomotion and also that cells preferred to align to major grooves of dimensions (36-78 micron) to the minor grooves. Sutherland et al. [112] have also observed in their studies that the degree of cell alignment in low-density cultures was dependent on both groove width and groove depth. Human dermal fibroblasts’ alignment increased with groove depth for all widths, while increase in alignment for all groove widths was most conspicuous for submicron depth devices. Contact guidance of primary stromal fibroblasts was found to be affected by both groove depth and pattern pitch when cultured in medium containing 10% serum (Figure 2.23) [113].

Figure 2.23 Contact guidance and morphology of primary stromal fibroblasts is influenced by groove depth and pattern pitch. Cell spreading on 265 nm and 880 nm deep grooves is shown in figures B and C respectively [113] (Reproduced with permission).
2.6. Cell migration on micropatterned substrates

Cell locomotion on the micropatterned substrates has been an area of interest in several research studies. Ultrathin fibronectin fibers of diameter 0.5-7 micron have been shown to promote Schwann cell migration with alignment of focal contacts and F-actin filaments along the axis of fibers [114]. Brunette et al. [57,71] found that grooves directed the migration of epithelial cells although the control was not absolute and cells crossed over the ridges and descended into the grooves. Clark et al. [84] showed that the migration of myoblasts was restricted in a direction parallel to the grooves.

Nanoscale topographical features have been shown to modulate migration of corneal epithelial cells. Deihl et al. [73] demonstrated that anisotropic substratum features promoted cell migration along their axes and inhibited cell migration orthogonal to their axes. An interesting conclusion was made by Hamilton et al. [55] indicating that a continuous edge is not a prerequisite for guided cell migration (Figure 2.24). Fibroblasts and epithelial cells migration was shown to be guided diagonally through gaps in the square patterns.

Madeja et al. [115] studied the effect of homotypic collisions and heterotypic interactions with normal human skin fibroblasts on the migration behavior of Walker carcinosarcoma cells. The tumor cells were shown to migrate along the long axes of underlying aligned fibroblasts and there was suppression in the directed migration by RGD-containing peptides. CNS neuroblasts’ peculiar perpendicular migration of CNS neuroblasts on topographical features was demonstrated by Nagata et al. [111] when topographical features had dimensions closed to those of tightly aligned neurite bundle. Schnell et al. [66] evaluated biodegradable fibers fabricated using electospinning of PCL.
and collagen and found that Schwann cell migration was significantly improved on collagen/PCL blend compared to pure PCL fibers.

Figure 2.24 Sequential time-lapse images of human gingival fibroblasts. Cells were cultured for 24 h prior to image collection. At 0 min, cell 1 has a square morphology, which subsequently becomes diagonally aligned by the Discontinuous edged surfaces (DES), by 180 min. At 360 min, the cell has migrated diagonally to the next box of the DES. Subsequently, the cell continues to migrate diagonally up until 900 min [55] (Reproduced with permission).

Hydrophobic surfaces have been shown to be more prone to direct cellular motility in comparison to hydrophilic surfaces. Su et al. [116] used a hydrophobic PDMS surface to study migration of fibroblasts and observed the sequential attachment of cells on surface, migration toward the walls, crossing over 30 micron high ridges. Turner et al. [67] showed that myogenic cells migrated along the parallel streaks of fibronectin. Wójciak-Stothard et al. [36] showed that the persistence of macrophage cell movement
on patterned substrata was significantly higher than that on plain substrata. Also, cells on shallow grooves moved with higher speed than cells on deep grooves.

![Image](image.png)

Figure 2.25 Time-lapse images of chondrocyte aggregation and guidance on (A) control flat silica, and on grooves of (B) 10 micron width and 3 micron depth and (C) 5 micron width and 10 micron depth. Two independent aggregates (1 and 2) collide (arrows) and form one larger aggregate at 240 min (3). The aggregate then migrates parallel to the groove long axis (arrow), with a collision imminent with aggregate 4 [110] (Reproduced with permission).

Wood et al. [117] demonstrated the migration of eleost fin mesenchyme cells on topographically patterned surfaces with individual cells becoming highly polarized. Hamiton et al. [110] made an interesting study on migration of monolayer and multilayer aggregates along the microgrooved surfaces. The tension generated by these aggregates propelled them forward in the direction of grooves (Figure 2.25). Anisotropic stiffness in substrate also effects the cell migration [102]. Cells were found to migrate along the direction of greatest rigidity. Poole et al. [103] found that the
structure of oriented collagen fibrils is a key parameter determining the motion of mouse dermal fibroblasts.

2.7. Cell bridging

With increasing groove depth and decreasing groove widths, cells tend to bridge across the ridges rather than descending into the grooves. Cell bridging can be used in conjunction with 3-D constructs to form oriented tissue like structures with desired mechanical strength and flexibility like that of normal tissue. Chehroudi et al. [118] observed the bridging behavior of epithelial cells on certain topographical patterns. The cells were attached on 1 micron and 3 micron deep horizontally and vertically aligned grooved surfaces, but in case of 22 micron deep horizontally oriented grooves, they bridged over the grooves. Ricci et al. [65] studied rat tendon fibroblasts on micropatterned substrates and observed that cells spanned over several grooves and ridges on 4 micron microgrooved substrates, while cells grown on the 12 micron microgrooved substrate are situated atop the ridges or within the grooves. He et al. [10] found that on third day of culture, rat fibroblast cells were able to bridge gaps form contact with cells aligned parallel to grooves. Goldner et al. [119] investigated the phenomenon of neurite bridging very extensively in their study on PDMS microgrooved surfaces (Figure 2.26). They reported a number of phenomena influencing the formation of bridging neurites. Groove widths, plateau widths and cell density were found to significantly affect neurite bridging. The researchers hypothesized that bridging occurs when neurites exert forces on each other that can override the effects of substrate features on neurite alignment.
2.8. Biological cues

Several studies have inferred that alignment of a type of cells stimulates alignment of second type of cells and thus facilitates in regeneration of tissues. This phenomenon has specifically been observed and investigated in neuronal regeneration which is enhanced by alignment of glial cells. Miller et al. [50] made an important inference that the presence of Schwann cells in the grooves of biodegradable polymer substrates promoted neurite alignment and outgrowth. Not only that, Schwann cells helped the neurites to orient even on shallower grooves and exhibited continued alignment even when the grooves degraded. Eguchi et al. [120] reported that an 8-T Magnetic field causes Schwann cell and collagen alignment, which facilitates neuronal regeneration. Deumens et al. [88] demonstrated the alignment of glial cells on biodegradable poly(d,l)-lactide matrices and thereafter cell-matrix complexes directed neurite growth. Alexander et al. [121] demonstrated that neurite outgrowth was enhanced significantly and directed consistently along the electric field-induced aligned astrocytes.

Thompson et al. [122] demonstrated that Neurite outgrowth can be directed by Schwann cell alignment in the absence of other guidance cues and suggested that this guidance is through both topographical and molecular mechanisms. Stepien et al. [123]
demonstrated the aligned growth of chick embryo neurons on underlying aligned human skin fibroblasts. Biran et al. [124] also observed the alignment of astrocytes on substrates containing unidirectional topographical features. These astrocytes enhanced the alignment of overlying neurites. Interestingly, the regenerating neurites consistently grew along the long axis of astrocyte alignment (Figure 2.27). At the borders of orthogonally aligned astrocytes, neurites turned to maintain their parallel trajectories. Sorenson et al. [91] have shown that micro-topography can act through an overlaid astrocyte layer and results in aligned neurites in long-term culture and that these can be myelinated by endogenous oligodendrocytes. Madeja et al. [115] reported directed migration in case of Walker carcinosarcoma cells when they were plated onto the surface of aligned fibroblasts.

Figure 2.27 Dorsal root ganglion (DRG) neurite outgrowth cultured atop nonaligned astrocyte monolayers on the 45-nm substrate (a) and aligned astrocyte monolayers on the 492-nm substrate. Scale bar: 100 micron. Astrocytes were visualized by immunostaining for GFAP (green) and neurons by beta-III-tubulin (red) [124] (Reproduced with permission).

2.9. Applications and scope

The grafting techniques for tissue repair have inherent defects like donor site morbidity, inferior physical, chemical and biomechanical characteristics and defective regeneration. Great emphasis has been placed in recent years upon fabrication of synthetic guidance conduits and scaffolds for tissue regeneration. An improved
understanding and better control of physical, chemical properties of several biocompatible polymers and their interaction with cells has resulted in major advances in achieving the goal of tissue regeneration.

The polymer conduits can play a crucial role in restoring cellular pathway and have great potential for tissue reconstruction. The first pioneering conduits were based on non-resorbable polymers like silicone and PTFE [125,126]. The practical limitations of these conduits like permanent fibrotic encapsulation of the implants and lack of functional recovery lead to development of conduits synthesized from bioresorbable polymers. PLA, chitosan, fibronectin, collagen and similar materials have been used to fabricate tissue repair guides [127-130].

One of the recent studies has compared the conduits with microgrooved inner surface to smooth conduits (Figure 2.28) and has shown that microgrooved surfaces can enhance peripheral nerve regeneration in vivo [131]. This can pave the way for a great leap in the field of tissue regeneration. The design rules gained from extensive research done for in vitro studies can be applied in designing similar in vivo implants to improve tissue regeneration strategies.

![Figure 2.28 Preparation of the microgrooved chitosan or PLA substrates and conduits [131] (Reproduced with permission).](image-url)
Hayashida et al. [132] designed an autologous valved conduit for implantation into circulatory system of living animals (Figure 2.29).

A wide variety of tissue scaffolds have been used to mimic the cellular microenvironment to facilitate tissue growth. Traditional methods to fabricate scaffolds usually do not result in uniform, well-arranged microstructures and the ability to control these features is extremely limited [133]. Recent developments in fabrication methods like solid free form fabrication have made it possible to fabricate scaffolds with well-organized features [134]. Well-defined scaffolds have been precisely fabricated and replicated in biopolymers like PLGA using novel combination of microembossing and carbon bonding (Figure 2.30) [135].
Papenburg et al. [7] combined micropatterning with fabrication of scaffolds having highly porous structures suitable for efficient transport of nutrients, oxygen and metabolic products and demonstrated their potential in building highly promising 3D scaffolds.

The design rules from in vitro studies on patterned surfaces can be employed in these well-defined scaffolds and this will facilitate cell attachment, spreading, proliferation, and differentiation and tissue generation. The control of cell behavior by various cues can provide us insight into the fundamental biological issues of how cells interact with the surrounding environment like cell-cell, cell-substrate, cell-ECM interactions. The understanding of fundamental biological phenomena, at molecular and cellular level, can be used to direct cell fates and manipulate cellular responses. The amalgamation of engineering approaches, with advances in material, medicine and biological research can potentially revolutionize regenerative and developmental biology.
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3.1. Introduction

Tendons are highly specialized connective tissues that connect muscles to bone and transmit tensile loads that move and stabilize joints. Tendon damage that occurs through repetitive strain or acute trauma is a major problem for the orthopedic biomedical community. Damaged tendons have a very limited capacity for regeneration, since these tissues are relatively avascular and sparsely populated with cells of low mitotic activity [1]. The ability of tendons to transmit tensile loads without structural failure is a consequence of the highly ordered arrangement of collagen fibers, aligned along the vector of primary load. When tendons are damaged, the resultant scar tissue is of inferior mechanical strength and elasticity. Conservative approaches to the treatment of tendon strain are currently focused on minimizing the acute inflammatory phase of repair and consequent scar deposition, with rehabilitation programs designed to optimize scar fiber orientation. Surgical treatments aim to stimulate vascular ingrowth and tissue repair, reduce peritendinous adhesion formation and restore tendon-bone continuity [2]. These current therapies do not reliably restore an optimally organized tissue for tensile load-bearing. Tendon tissue engineering offers a promising alternative to regenerate damaged tendons. However, the factors that have the ability to stimulate tendon repair have not been identified.

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Controlling cell-substrate interactions is critical to developing successful tissue engineering strategies. Recent advances in lithographic methods for microfabrication have facilitated substrate patterning and modification for cell studies. Researchers have studied the effect of surface topography [3,4], surface crystallinity [5], hydrophobicity [6], surface roughness [7], and chemical composition [8] on cellular responses. Cell substrate interactions have been studied in the context of patterned surface substrates for many cell types: fibroblasts [9-11], BHK cells [12,13], neuronal cells [14], Schwann cells [15-17], macrophages [18], epithelial cells [19], endothelial cells [20], and smooth muscle cells [21,22].

To date, the majority of studies addressing tendon tissue engineering have focused on developing biocompatible polymeric scaffolds for the directed migration of endogenous reparative cell populations [23,24] or ex vivo colonization of isolated cells prior to surgical implantation [25-27]. Mechanical loading is required for tenocyte homeostasis [28,29] and also impacts intrinsic tendon repair [30,31]. Undoubtedly, biomechanical stimuli have an influence on the performance of any tissue-engineered tendon construct, but successful tendon regeneration will require a comprehensive understanding of the fundamental biological issues governing tenocyte growth and matrix orientation.

Tenocyte alignment and appropriate orientation of newly synthesized matrix are critical for tendon function [23, 32]. However, the stimuli that influence the alignment of cells and the extent to which these factors affect tenocyte behavior are not yet well understood. In this work, we addressed the role of microtopographical factors on the
alignment and growth of tenocytes. We fabricated substrates with groove widths ranging from 50 to 250 µm. These patterned substrates were used to investigate the influence of physical cues on the proliferation, cytomorphology, alignment and gene expression of tenocytes.

3.2. Materials and methods

3.2.1. Micropatterned substrate fabrication

Glass slides, 25 mm x 75 mm x 1 mm, (Fisher Scientific, Pittsburgh, PA) were used as substrates for the fabrication of micropatterned structures. The slides were cleaned in Piranha solution (H₂SO₄: H₂O₂ (30%) = 3:1) overnight, and subsequently rinsed with DI water and dried in nitrogen. This was followed by sonication in isopropanol and acetone for 5 minutes each. After drying with nitrogen, the slides were exposed to oxygen plasma (100 mm Torr, 100 W power) for 1 minute. The micropatterned substrates were fabricated on the cleaned glass slides using standard photolithographic procedures. SU-8 5 photoresist (Microchem, Newton, MA), was spin-coated on the glass slides at 1000 rpm for 30 s. SU-8 is a biocompatible photoresist obtained by dissolving a polymeric epoxy resin (glycidyl ether of bisphenol A) in an organic solvent (gamma-butyrolactone) and adding a photoacid generator taken from the family of the triarylium-sulphonium salts [33]. The substrates were soft-baked at 65 °C for 2 minutes, followed by baking at 95 °C for 5 minutes. Transparency masks (CAD/Art services, Bandon, OR) of the pattern were placed on the glass substrates and exposed to UV light using a mask aligner (Suss Microtech MJB3) for 12 s (power = 334 MW). The substrates were then subjected to a two-step post-exposure bake: 1 minute at 65 °C followed by 2 minutes at 95 °C. The patterns were developed in a PGMEA solution
(Sigma-Aldrich, St. Louis, MO) by dissolving the unexposed portions of the photoresist. After drying the substrates with nitrogen, they were exposed to UV light without a mask and hard baked at 150 °C on hotplate overnight, to ensure complete polymerization of SU-8 and enhance its adhesion to the glass. The micropatterned slides were sterilized with hydrogen peroxide before use in cell culture experiments and were treated with oxygen plasma to promote cell adhesion. A schematic illustration of the micropatterned substrate is shown in Figure 3.1a, along with a high-resolution of a 50 µm pattern (Figure 3.1b). The grooves of the micropatterns were free from photoresist all the way down to glass.

Figure 3.1 (a) Schematic illustration of micropatterned substrates with critical dimensions; groove width (W): 50 – 100 – 250 µm; ridge width (D): 50 µm; and ridge height (H): 15 µm. (b) Scanning Electron Micrograph (SEM) image of a 50 µm grooved micropatterned substrate. Scale bar: 50 µm. (Reproduced with permission).
3.2.2. Tenocyte isolation and culture

Superficial digital flexor tendon specimens were collected from four adult horses that were euthanized for reasons not associated with musculoskeletal disease. These horses were euthanized in accordance with approved IACUC protocols by an intravenous overdose of a barbiturate anesthetic agent. The tendon specimens were diced and digested with trypsin-EDTA for one hour (Invitrogen, Carlsbad, CA) followed by overnight digestion in 0.15% collagenase II (Worthington, Lakewood, NJ). The trypsin and collagenase digestions were carried out in a 37 °C shaking incubator. Following overnight incubation, the digest suspension was passed through a 40 µm pore-size filter to remove incompletely digested tissues. The released cells were pelleted by centrifugation at 390 g for 10 minutes and washed twice in phosphate-buffered saline (PBS; HyClone, Logan, UT). The cells were counted using a hematocytometer and cryo-stored in freezing medium containing 50% DMEM high glucose (HyClone, Logan, UT), 40% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 10% DMSO (Fisher Scientific, Rochester, NY) in liquid nitrogen.

3.2.3. Tenocyte seeding onto the patterned surfaces

The tenocytes were thawed, counted and seeded in 55 cm² culture dishes (Corning Incorporated, Corning, NY) at a density of 5 x 10⁴ cells/cm². They were cultured in high glucose DMEM, supplemented with 10% fetal bovine serum, L-Glutamine (2 mM) (Invitrogen, Carlsbad, CA), penicillin G sodium (100 units/ml) - streptomycin (100 µg/mL) (Invitrogen, Carlsbad, CA), amphotericin B (2.5 µg/ml) (MP Biomedicals, Solon, OH) and ascorbic acid (50 µg/ml) (Wako, Richmond, VA) until confluence. The cells were then trypsinized and seeded onto the patterned surfaces at an initial density of 5 x
10^4 cells/cm^2 in 4-well rectangular dishes (Thermo Fisher Scientific, Rochester, NY) and cultured under 5% CO2 at 37°C for up to 72 hours. The seeding density calculations were based on the surface areas of the microgroove floors available for cell attachment. Caution was taken to ensure that cells were not seeded in clumps thus ensuring uniform distribution. In each experiment, ten replicates were seeded for each micropatterned substrate and time point: two patterns for confocal microscopy, two for SEM, two for optical microscopy, and four patterns for RNA isolation.

Optical microscopic images of the cells were acquired with a Nikon Eclipse TS 100 inverted microscope on days 1, 2 and 3 after seeding. The day 1 image acquisition occurred between 16 and 20 hours after seeding, before any substantial cell division could occur. Samples were collected for gene expression analyses at 36 and 72 hours. In addition, slides were placed in 10% buffered formalin (Fisher Scientific, Rochester, NY) for fluorescent confocal analyses of cell density and cytoskeletal organization. These slides were collected at 16 hours after seeding, prior to any cell proliferation, at the midpoint of the study (36 hours) and at the termination of study (72 hours).

3.2.4. Confocal and fluorescent imaging

The formalin-fixed slides were washed three times with PBS. The cells were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) at room temperature for 5 minutes, and then washed again in PBS. The slides were blocked with 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS for 20 minutes to prevent non-specific binding. Alexa Fluor® 488 phalloidin (Invitrogen, Carlsbad, CA) was reconstituted in methanol, diluted in PBS to 5 U/ml and applied to the slides for 20 minutes. The slides were rinsed in PBS, exposed to 0.1 μg/ml of DAPI in PBS,
(Invitrogen, Carlsbad, CA) for 1 minute, washed in PBS and cover slip-mounted using an aqueous mounting medium (R&D Systems Inc., Minneapolis, MN). Cells were imaged with a Leica TCS SP2 multiphoton confocal laser scanning microscope (Leica Microsystems, Germany). The images were acquired with 10x (0.4 NA), 40x (1.25 NA) and 63x (1.32 NA) objectives. The Alexa 488 dye was excited using the 488 line of an Argon laser. DAPI fluorescence was detected under the two-photon excitation with a 785 nm pulse laser line (Ti:Sapphire, 100 fs, 80 MHz). Polarized light microscopy was used to image collagen fibril deposition and organization in formalin-fixed specimens, using a Leica DM 2000 light microscope and polarizing lens. Images are acquired using a Leica DFC320 digital camera.

3.2.5. Cell density calculations

The fluorescent confocal images of cell layers were used for cell density calculations. DAPI-stained cell nuclei were counted in twenty randomly selected 12500 µm² regions of interest (ROI) across the floors of the micropatterned grooves. Cell densities were extrapolated from an average of the nuclear counts per ROI area.

3.2.6. Quantitative analyses of cell shape and alignment

Images of the tenocytes cultured on micropatterned surfaces were quantitatively analyzed using ImageJ software (free download available at http://rsbweb.nih.gov/ij/). Briefly, the images acquired from optical microscope were converted to 8-bit grey scale and thresholded to distinguish cellular outlines from non-cellular background signal. Thus, the program detected cells on the basis of contrast and fitted the cellular outlines to equivalent ellipses [22,34]. Areas smaller than the area of a cell were excluded while fitting the ellipses. The following cell-shape characteristics were measured for each
fitted ellipse: major axis, minor axis, aspect ratio (major axis/minor axis), perimeter, area and orientation angle with respect to the direction of grooves. The major axis was taken as the direction of angle of orientation of the cells. The angle of the micropattern was measured for each image and added or subtracted from the orientation angle of the tenocytes to get an accurate value for the orientation of cells with respect to the grooves. The angle for each cell was converted such that 0° represented cell orientation along the direction of the microgroove and 90° represented a perpendicular orientation with respect to the direction of the grooves. The data for each image were transferred into Excel (Microsoft, Seattle, WA) for further analysis. For more detailed evaluation of cell orientation in the 250 µm microgrooves, the substrate surface was segregated into ‘edge’ zones within 50 µm of the groove wall, ‘mid’ zones positioned between 50 and 100 µm from the groove walls and ‘central’ zones located in the central 50 µm of the microgrooves.

Cell morphology was quantified using cell shape index (CSI), defined as \((4\pi \times \text{cell area})/\text{(cell perimeter squared)}\). The cell shape index is a measure of cell roundness, with ‘1’ representing a perfect circle and ‘0’ a straight line. The cellular contours were identified and analyzed for all non-overlapping cells in this manner. In cases where confluent cell layers prevented automated assessment due to cell-cell contact, cellular outlines were manually drawn using a Graphire Pen Tablet (Wacom Technology Corporation, Vancouver, WA) and orientation angles and other variables were measured for each cell individually using ImageJ. Cells that overlapped or aggregated together or wherever boundaries of contacting cells could not be distinguished clearly
were not used for quantitative analysis. Two hundred cells were used for quantitative analysis for each micropattern for each time point.

3.2.7. Gene expression analyses

Gene expression analyses were carried out using tenocytes isolated from four donors, to determine whether microtopographical cues influence the transcription of genes associated with proliferation, matrix synthesis or the tenocytic phenotype. To this end, we compared gene expression of tenocytes seeded on substrates with 50 µm microgrooves and 250 µm microgrooves, to reflect tightly constrained and unconstrained attachment conditions, respectively. Gene expression was assessed at the mid-point (36 hours) and the termination (72 hours) of the study.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
<th>size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>5’CATGAACTACCTGGACCAGCCT</td>
<td>5’TCGAAAGGACAGGAAGTTGTT</td>
<td>438</td>
</tr>
<tr>
<td>Cyclin A2</td>
<td>5’TGAAGATGAAAGCCAGTGA</td>
<td>5’GAGGTAGCTCTGGTGAGTC</td>
<td>704</td>
</tr>
<tr>
<td>PCNA</td>
<td>5’GTGTAACCTGCAGACATG</td>
<td>5’GGAATTCCAAGTTGTTCAAC</td>
<td>285</td>
</tr>
<tr>
<td>Coll I</td>
<td>5’AGCCAGCAGATCGAGAACAT</td>
<td>5’CGCCATACTCGAACTGGAAT</td>
<td>303</td>
</tr>
<tr>
<td>Coll III</td>
<td>5’AGGGGACCTGGTACTGCTT</td>
<td>5’TCTCTGGTGGGACAGATCT</td>
<td>215</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>5’GACGCCGAGAGCGAAGGTGT</td>
<td>5’AAGAAGTTGTCGGGCTGTT</td>
<td>201</td>
</tr>
<tr>
<td>COMP</td>
<td>5’TGTGTGGAAGCAGATGGAG</td>
<td>5’TAGGAACCAGGGTGGAGTAGATG</td>
<td>224</td>
</tr>
<tr>
<td>Tendin</td>
<td>5’CCCTCAAGTGGAAGGTGGAGA</td>
<td>5’GTTGCAAGGCGATGACAC</td>
<td>149</td>
</tr>
<tr>
<td>EF1-alpha</td>
<td>5’CCCGGACACAGGACTTCCAT</td>
<td>5’AGCATGGTGGTACCATTCCA</td>
<td>329</td>
</tr>
</tbody>
</table>

Total RNA was extracted from tenocytes using Trizol® (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. Northern Blot analyses were carried out using
previously described protocols [35,36] to assess the expression of the following strongly expressed genes: collagen type I, proliferating cell nuclear antigen (PCNA) and elongation factor 1-alpha (EF1-alpha). The primers used to generate the cDNA probes are listed in Table 3.1.

Briefly, 4 µg of total RNA was electrophoresed through an agarose-formaldehyde gel. After a 2-3 hour separation, the ribosomal RNA bands were stained with ethidium bromide to assess equivalence of loading and RNA integrity. The RNA was then transferred to a charged nylon membrane (GE Osmonics Labstore, Minnetonka, MN) by high-salt capillary transfer, and cross-linked by ultra-violet light. The membranes were rinsed in 5x SSPE and pre-hybridized with a hybridization solution containing 5x SSPE, 5x Denhardtts’ solution (Eppendorf, Westbury, NY), 0.5% SDS (Fisher Scientific, Rochester, NY), 10% dextran sulfate (Molecular Weight 5 x 10^5) (Fisher Scientific, Rochester, NY), and 150 µg/ml of denatured salmon sperm DNA (Invitrogen, Carlsbad, CA) for one hour. The probes were radiolabeled with dCTP ^32P (Amersham, Piscataway, NJ) using Prime-It II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX) following manufacturer’s instructions, denatured and then added to tubes containing the membranes that had been pre-hybridized. After overnight hybridization, the membranes were washed in 2x SSPE containing 0.5% SDS. Sequential washes were done until the washing solution reached 0.5x SSPE with 0.125% SDS. The membranes were wrapped in plastic and placed into a lightproof cassette with radiograph film and stored at -80 °C during exposure. The radiograph film was developed after 1-4 days of exposure, depending on the target mRNA abundance and probe activity.
Quantitative PCR (qPCR) was simultaneously used to compare expression profiles of tenocytes seeded onto 50 µm and 250 µm substrates, representing constrained and unconstrained conditions, respectively. Expression patterns of the extracellular matrix genes collagen type I, type III and aggrecan, the phenotypic markers Cartilage Oligomeric Matrix Protein (COMP) and tendin, the proliferation markers PCNA, Cyclin D1 and Cyclin A, and the reference gene elongation factor 1-alpha (EF1-alpha) were assessed in these experiments. One microgram of total RNA from each sample was converted to cDNA using oligo T primers and Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The samples were run in duplicate and each reaction (25 µl) contained 12.5 µl of SYBR® GreenER™ qPCR super mix (Invitrogen), 10 pMol of sense and anti-sense primers (Sigma-Aldrich, St. Louis, MO) and 25 ng cDNA. SYBR Green fluorescence was measured using a Bio-Rad 'iCycler' thermal cycler (Bio-Rad Laboratories, Hercules, CA). The expression data in each experiment were normalized to expression of the reference gene, EF1-alpha. For each target gene, relative mRNA levels were converted to fold changes in expression relative to expression in the 50 µm-36 hr samples. As an example, if the mean expression of the target gene at 72 hours was twice that of the 50 µm-36 hour measurement, the fold-increase would be designated as ‘2’.

3.2.8. Statistical analyses

Statistical differences between cell densities and cell shape indices were analyzed by two-way ANOVA (p<0.05). Dunnett’s or Bonferroni’s post hoc test were used as required to identify significant differences between specific data sets. The Kolmogorov-Smirnov (KS) test was used to assess the significance of differential alignment
behaviors of tenocytes on the substrates (p<0.05). qPCR data were also analyzed by two-way ANOVA (p<0.05), using data sets from four replicate experiments.

3.3. Results

Preliminary experiments were carried out using micropatterned substrates with groove widths ranging from 20 to 500 µm, to identify biologically informative microgroove dimensions. Tenocytes seeded onto substrates with 20 µm grooves were not able to attach efficiently, while cell monolayers seeded onto microgrooves 250 µm or wider closely resembled unconstrained monolayers. Therefore, subsequent experiments focused on the differential behavior of tenocytes on substrates with 50 µm and 250 µm wide microgrooves, representing highly constrained and unconstrained seeding conditions, respectively. In some experiments, 100 µm wide microgrooved substrates were included to represent an intermediate degree of confinement. The controls comprised of an unpatterned glass surface providing a completely unrestricted environment for cell growth and proliferation. Cell attachment was also assessed on SU-8 surfaces. SU-8 has been used as a patterning material in substrates in fabrication of bioreactors and is non-cytotoxic [37]. Successful cell attachment, growth and proliferation on SU-8 patterned surfaces demonstrated the biocompatibility of SU-8 photoresist.

3.3.1. Effects of microgroove width on cell density

Tenocytes were seeded at an initial density of 5 x 10⁴ cells/cm² onto 50 µm, 100 µm, and 250 µm wide microgrooves or onto an unpatterned substrate. The seeding densities were calculated on the basis of the groove ‘floor’ areas only and were selected to provide for approximately two cell doublings on each surface prior to confluence. At
the first assessment point, within the first day of seeding and prior to any significant proliferative activity, no changes in cell density were observed (Figure 3.2).

By 36 hours, the growth curves had diverged and there were significant differences in cell densities between all four substrate groups by 72 hours (Figure 3.2). The cell densities at confluence reflected the degree of constraint. Accepting the statistical outcomes, it was apparent that the cell density at confluence in the 50 µm grooves was substantially less (approximately half) than those of the other three substrates.

![Figure 3.2 Cell density as a function of time after seeding.](image)

3.3.2. Effects of microgroove width on cell shape

Quantitative analyses of tenocyte shape after seeding onto confined (50 µm) or unconfined (250 µm) microgrooves demonstrated that both populations became
progressively and significantly more fusiform (long and narrow) over time, as the cells reached confluence (Figure 3.3).

Figure 3.3 Cell shape index as a function of time after seeding. a, b and c indicate statistically significant differences between cell shape indices across time on 50 µm patterned substrates. Similarly, A, B, and C indicate statistically significant differences across time on 250 µm patterned substrates. The asterisks * denote statistically significant differences between the substrate groups at each time point (n=30; p<0.05) (Reproduced with permission).

Prior to confluence, one and two days after seeding, the tenocytes seeded under constrained conditions (50 µm) were significantly more fusiform than unconstrained cells (250 µm). However, by day 3, once cell density peaked, the cell shape indices were highly similar.
Figure 3.4 Confocal images of tenocytes growing on 50 µm (a and c), and 250 µm (b and d) micropatterned substrates. Polymeric actin is localized with phalloidin (green), while cell nuclei are localized with DAPI (blue). Tenocyte monolayers 36 hours after seeding onto grooves of widths of (a) 50 µm, and (b) 250 µm. Higher power images of individual cells seeded onto (c) 50 µm and (d) 250 µm substrates. Scale bar: 50 µm. (Reproduced with permission).

As seen in Figure 3.4a and Figure 3.4b, the majority of cells in each group adopted a fusiform morphology (see also Figure 3.5a-d). However, under confined conditions (50 µm), this morphology was consistent (Figure 3.4c), whereas the unconstrained conditions provided by 250 µm microgrooves accommodated a small number of comparatively stellate cells prior to confluence (Figure 3.4d).
Figure 3.5 Alignment of tenocytes on micropatterned substrates. Images show representative tenocyte orientations in grooves of widths (a) 50 µm, (b) 100 µm, (c) 250 µm, and on (d) unpatterned control slides, on day 1. Scale bar: 50 µm. Plots show the cumulative angular distribution fractions of tenocytes (Y-axis) in 1° bins (X-axis) (e) one (f) two (f) and (g) three days after seeding. In all cases, 0° corresponds to the direction along the grooves. The plots are based on measurements using 200 cells for each substrate pattern at each time point. In each plot, significantly different curves are designated by distinct letters, A, B, C or D (Reproduced with permission).
3.3.3. Effects of microgroove width on cell alignment

Microgroove width exerted a profound effect on cell alignment. As expected, tenocytes seeded onto 50 µm microgrooves were aligned within 30° of the microgroove orientation soon after seeding (Figure 3.5a and 3.5e). The cells on unpatterned surfaces exhibited an essentially random orientation, while tenocytes seeded onto 100 µm and 250 µm microgrooves showed intermediate alignment (Figure 3.5b-g). After 3 days in culture, the alignment of tenocytes seeded onto 50 µm microgrooves was more focused along the orientation of the grooves, with 50% of the population aligned within 5° of the groove axis and the entire population constrained within 20° of this vector (Figure 3.5g). As the cell layers reached confluence, the tenocyte alignment distribution within the 100 µm microgrooves closely approximated that of the 50 µm group (Figure 3.5f and 3.5g). Cell alignment on the unpatterned surfaces remained random, while the cells seeded onto 250 µm microgrooves displayed an intermediate level of orientation, with 50% of the cells aligned within 20° of the groove axis (Figure 3.5g). This outcome was driven, to a large extent, by the edge effect exerted on the confluent cell layers by the groove walls. Cells adjacent to groove walls (‘edge’ zone) were aligned as a result of guidance provided by the walls, whereas cells positioned in the ‘mid’ and ‘central’ zones of the groove were significantly less aligned to the longitudinal axis of the groove (Figure 3.6). ‘Central’ zone cells were, in effect, randomly aligned, as was evident on the unpatterned surfaces (Figure 3.5g and 3.6b).
Figure 3.6 (a) Angular distribution of tenocytes as a function of distance from groove wall. The region within each 250 µm width micropatterned groove is divided into three zones transversely in order to determine the edge effects: ‘edge’ (0-50 µm from groove wall), ‘mid’ (50-100µm) and ‘central’ (100-150 µm) regions. (b) The distribution plot shows the cumulative angular distribution fractions of tenocytes (Y-axis) in 1° bins (X-axis) within each zone one day after seeding. The plots are based on measurements using 150 cells for each zone in 250 µm wide substrate pattern. The significantly different curves are designated by distinct letters, A, B, or C. Scale bar: 50 µm. (Reproduced with permission).
3.3.4. Effects of microgroove width on collagen alignment

The alignment of secreted collagen was assessed using polarized light microscopy. The birefringence of collagen secreted by tenocytes seeded onto 50 µm microgrooves (Figure 3.7b) was noticeably stronger (pink/orange bands) than the signals detected from unseeded microgrooves (Figure 3.7a) or from tenocytes on 250 µm substrates (Figure 3.7c) and appeared to be organized in a sinusoidal pattern, bounded by the groove edges. In contrast, the samples with 250 µm microgrooves exhibited little or no collagen fiber birefringence.

Figure 3.7 Polarized light microscopic images of (a) an unseeded 50 µm wide patterned substrate and of collagen secreted by tenocytes seeded onto (b) 50 µm, and (c) 250 µm wide patterned substrates after 72 hours. Scale bar: 50 µm. (Reproduced with permission).

3.3.5. Effects of microgroove width on gene expression

The gene expression analyses focused on the differential effects of 50 µm and 250 µm patterned substrates on tenocytes and are presented in Table 3.2. Time in culture exerted significant but opposite effects on the expression of genes associated with cell proliferation (Figure 3.8; Table 3.2) and extracellular matrix synthesis. Expression of PCNA (Figure 3.8), Cyclin D1 and Cyclin A2 (Table 3.2) fell significantly between days 1
and 3, indicative of transient proliferative activity prior to the cell layers reaching confluence. In contrast, expression of collagen types I (Figure 3.8) and III, and aggrecan (Table 3.2) increased in the later stages of culture, although considerable inter-experimental variability in aggrecan expression precluded a statistically significant result in this study. The influence of substrate groove width on expression of the tenogenic markers Tendin and COMP was not marked. Tendin mRNA levels fell moderately over time, while COMP expression was increased by approximately 50% in the 250 µm group.

Table 3.2 Results of tenocyte gene expression analyses

<table>
<thead>
<tr>
<th></th>
<th>36 hrs</th>
<th>72 hrs</th>
<th>P values</th>
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<tr>
<td></td>
<td>50 µm</td>
<td>250 µm</td>
<td>50 µm</td>
<td>250 µm</td>
<td>Time</td>
</tr>
<tr>
<td>Proliferation Markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>1.01 ± 0.172</td>
<td>0.64 ± 0.227</td>
<td>0.07 ± 0.014</td>
<td>0.42 ± 0.246</td>
<td>0.0002</td>
</tr>
<tr>
<td>Cyclin A2</td>
<td>1.01 ± 0.172</td>
<td>0.91 ± 0.260</td>
<td>0.09 ± 0.031</td>
<td>0.14 ± 0.120</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PCNA</td>
<td>1.00 ± 0.072</td>
<td>1.34 ± 0.348</td>
<td>0.21 ± 0.066</td>
<td>0.19 ± 0.022</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ECM genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coll I</td>
<td>1.01 ± 0.154</td>
<td>0.79 ± 0.08</td>
<td>1.91 ± 0.23</td>
<td>2.39 ± 0.90</td>
<td>0.0002</td>
</tr>
<tr>
<td>Coll III</td>
<td>1.01 ± 0.128</td>
<td>1.13 ± 0.052</td>
<td>2.04 ± 0.167</td>
<td>1.97 ± 0.219</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>1.09 ± 0.523</td>
<td>0.75 ± 0.196</td>
<td>0.81 ± 0.384</td>
<td>2.86 ± 2.028</td>
<td>0.1113</td>
</tr>
<tr>
<td>Tenogenic genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tendin</td>
<td>1.02 ± 0.206</td>
<td>1.12 ± 0.109</td>
<td>0.9 ± 0.024</td>
<td>0.74 ± 0.095</td>
<td>0.0023</td>
</tr>
<tr>
<td>COMP</td>
<td>1.01 ± 0.146</td>
<td>1.12 ± 0.125</td>
<td>1.20 ± 0.156</td>
<td>1.61 ± 0.265</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

*In each experiment, the level of expression of the genes listed in this Table was normalized to the expression of the reference gene, EF1-alpha. For each gene, relative mRNA levels are presented as fold changes in expression relative to expression in the 50 µm-36 hr samples in each experiment. Mean ± Standard deviation; n = 4.*
Figure 3.8 Northern blot of proliferating cell nuclear antigen (PCNA), collagen type I (Coll I) and elongation factor 1- alpha (EF1-alpha) expression by tenocytes cultured on 50, 100 and 250 µm patterned substrates for one and three days (Reproduced with permission).

3.4. Discussion

3.4.1. Cell shape characteristics on topographically patterned substrates

The effect of topographical patterning on tenocyte shape was studied quantitatively and qualitatively using static optical micrographs and confocal images. Accepting that the cell shape indices of tenocytes seeded onto 50 µm substrates were statistically lower than cells growing in 250 µm grooves after one and two days in culture, reflecting a more fusiform morphology, this effect was not marked. The quantitative differences at these times amounted to less than 10% of the total index that encompasses cell shapes ranging from circles (1.0) to virtual lines (0.0). The tenocytes were predominantly fusiform on both substrates, and this morphology was progressively re-enforced over time, as the cell layers became confluent. By day 3, the cell shape indices were essentially identical (Figure 3.3).

Although it was not subject to quantitative assessment, the fluorescent, confocal images of tenocytes in Figure 4a-d demonstrate that substrate microtopography also affected nuclear profiles. The nuclei of cells seeded onto 50 µm microgrooves were
more ovoid than those of cells seeded under unconstrained conditions. This feature is particularly evident in the “single cell” images in Figure 3.4c and 3.4d. Nuclear deformation has been linked to alterations in tenocyte signal transduction [38] and constitutes a potential mechanism by which substrate microtopography might influence tenocyte behavior independently of direct cellular orientation or growth constraints.

3.4.2. Effects of microtopography on tenocyte alignment

Microgroove widths significantly influenced tenocyte alignment, consistent with the outcomes of a number of other studies that addressed cell-topography interactions using cells of neuronal, epithelial and myoblastic lineages [12,39-43]. The impact of microgroove width was evident within the first day of seeding. Approximately 65% of tenocytes seeded onto 50 µm microgrooves were aligned within 10° of the groove axis and 90% were within 20° of the axis, at the first assessment. The degree of alignment increased over time; almost all cells in 50 µm and 100 µm grooves were aligned along the channel axis after 72 hours of seeding.

In contrast to the tenocytes seeded onto 50 µm and 100 µm grooves, the cells seeded onto 250 µm grooves exhibited near-random orientation (Figure 3.5f). Accepting this, it was apparent that tenocytes growing under essentially unconstrained conditions developed locally aligned cell clusters at confluence, although there was no overall alignment of the cell population. This observation, along with the progressive orientation that occurred in the 50 µm and 100 µm-confined tenocytes over time (Figure 3.5e and 3.5f), suggest that intercellular interactions play a role in positioning individual cells, independently of any constraints imposed by substrate geometries.
Not surprisingly, the microgroove walls exerted a considerable local effect on tenocyte alignment. This was evident immediately in the 50 µm microgroove populations and became progressively more influential in the 100 µm and 250 µm cultures, over time (Figure 3.5e-g). In effect, cells positioned directly along the microgroove walls applied an alignment cue to adjacent cells as the monolayers reached confluence. This phenomenon was responsible for the late-stage quantitative differences between the tenocyte populations seeded onto 250 µm grooves and those seeded onto unpatterned surfaces (Figure 3.5g), and was clearly evident in the analyses of cell alignment in the ‘edge’, ‘mid’ and ‘central’ regions of the 250 µm grooves (Figure 3.6). The critical effects of substrate interfaces on cell behavior have been recognized in a number of other studies [17,39] and represent an additional mechanism to influence cell alignment, independent of other substrate geometries.

In recent years, a number of promising technologies have been developed to address tissue engineering applications of highly anisotropic tissues such as tendon, ligament and peripheral nerves [44,45]. In these tissues, cell orientation, and associated matrix alignment is critical for subsequent function. In particular, surface lithographic and electro-spinning techniques have been used to generate highly oriented scaffolds, where scaffold nano-topographies are considerably less than cellular dimensions [24,41,44,46]. The phenotypic consequences of substrate-mediated cellular alignment have varied considerably between studies, as summarized in a review by Martínez et al. [47]. The results of our study indicate that tenocyte alignment can be induced by topographic constraints that are significantly larger than individual cellular dimensions, consistent with the findings of other studies [48]. These physical cues can
be easily incorporated into the surface topographies of solid-state planar scaffolds to regulate alignment of ex vivo-seeded cells or control orientation of reparative cells that colonize scaffolds following implantation. The pronounced “edge effect” observed at the periphery of the 250 µm microgrooves (Figure 3.6) suggests that topographical cues such as distinct surfaces or material interfaces can exert considerable effect on cells without explicitly requiring a confined groove.

3.4.3. Effects of microtopography on collagen orientation

Of particular importance to the issue of tendon regeneration, polarized light microscopic imaging of the extracellular matrix secreted by the tenocyte monolayers demonstrated a sinusoidal organization of collagen fibers (Figure 3.7a) in the 50 µm-confined cultures. The periodicity of this organization (80-100 µm) approximates that of the collagen fiber “crimp” seen in equine flexor tendons [49] and rat tail tendons [50]. The matrices secreted by tenocytes maintained in 250 µm microgrooves showed no evidence of collagen alignment (Figure 3.7b). The organization of tendon collagen provides the 3-5% tensile elasticity critical for the load-bearing function of mature tendons and is a structural requirement for effective functional regeneration of tendon tissue. The sinusoidal collagen fiber organization observed in the 50 µm microgrooves developed in the absence of any mechanical loading stimulus, apart from the intrinsic tension generated through cell adhesion to the underlying substrates. These data are consistent with similar experiments carried out with MC3T3-E1 osteoblast-like cells [51] and suggest that appropriate alignment of secreted collagen can occur as a consequence of primary cell orientation, independent of load [52]. Additional
experiments will be required to determine whether cell alignment and tensile loading exert synergistic effects on tenocyte matrix synthesis and organization.

3.4.4. Effects of microtopography on tenocyte gene expression

Overall, the gene expression analyses documented a time-dependent transition from a proliferative cell population to a growth-arrested, matrix-synthetic population, as evident in the time-dependent down-regulation of PCNA and cyclin expression and concurrent increases in matrix gene mRNA levels. Substrate microtopography had comparatively little influence on this transition; most likely reflecting the equal seeding densities at the start of each experiment (Figure 3.2) and subsequent impact of contact inhibition as the cell monolayers approached confluence.

The phenotypic responses of tenocytes to the micropatterned substrates were inconsistent. Tendin mRNA levels were down-regulated over time on both substrates, whereas COMP expression increased significantly with time in culture and was also more highly expressed in the unconstrained (250 µm) tenocyte cultures (Table 3.2). COMP acts to regulate collagen fibrillogenesis in load-bearing connective tissues [53] and COMP expression by tenocytes is stimulated by both mechanical and growth factor-mediated anabolic stimuli [54]. Increased COMP expression by tenocytes in the later stages of these experiments likely reflects the matrix-synthetic state of the cells at these times. Collectively, there was no indication that cell and collagen matrix alignment augmented expression of the tenocytic phenotype, consistent with recent findings of Charest et al. in the context of myoblastic differentiation [43].

The effects of substrate topography cannot be separated completely from the effects due to patterned chemistry. The control experiments, however, revealed that less than
10% of cells preferred to attach to SU-8 ridges compared to glass microgrooves. Subsequently, the degree of confined environment controlled alignment of cells within the grooves. Therefore, the quantitative analysis presented here focused on influence of substrate dimensions on alignment response of cells and potential effects of surface chemistry were not considered. In future work, it would be instructive to compare synergistic as well as competitive effects of surface chemistry and topography by using chemically modified surface that shows comparable cell adhesion.

Design rules, such as those derived from the *in vitro* studies reported here, can be applied in the design of similar *in vivo* implants to improve tendon regeneration strategies [55]. Recent studies comparing conduits with microgrooved inner surfaces to smooth-surfaced conduits have shown that microgrooved surfaces can enhance peripheral nerve regeneration *in vivo* [45,56,57]. The results of the current study indicate that analogously designed conduits with optimum dimensions could be developed to improve tendon repair. Further, a number of additional factors can govern cell behavior and cell-substrate interactions, including substrate properties, presence of growth factors, and mechanical loading. All these have to be considered individually and collectively when designing scaffolds with implantable materials for *in vivo* studies. Our results provide a basis for geometric considerations in designing microgrooved scaffolds for tendon repair.

**3.5. Conclusions**

Micropatterned surfaces can direct the aligned growth of tenocytes. We demonstrated this phenomenon of contact guidance by microfabricated patterns created by simple photolithographic methods. After three days in culture, 99.5% of tenocytes
were aligned within 20° of the direction of grooves in 50 µm micropatterns. Groove
widths also significantly influenced cell shape and growth. The cells were more
elongated and less dense at confluence in narrow grooves. The most significant finding
was the influence of cell alignment on secreted collagen fiber alignment. Even in
absence of mechanical loading, collagen alignment was observed in correspondence
with cellular alignment. Proliferative and matrix gene expression profiles were inversely
affected by time in culture, and were minimally influenced by surface topography.
Further, surface topography did not influence expression of the tenocytic phenotype
markers, Tendin and COMP. In summary, microtopographical features can be optimally
controlled to influence both tenocyte and extracellular collagen alignment for tissue
engineering applications.

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Chapter 4 Neutrophil chemotaxis: literature review

4.1. Introduction

The phenomenon of directed migration of cells along chemical gradients is called chemotaxis. It is an important mechanism in the immune system as well as in many aspects of tissue development and maintenance. In response to an injury, immune cells are recruited to fight off infecting microbes. A number of chemicals, called chemoattractants, are produced at or proximal to sites of infection and inflammation and then diffuse into the surrounding tissue. Immune cells sense these chemoattractants and move in the direction where their concentration is greatest, thereby locating the source of the attractants and the associated targets. Leading the assault against new infections is a specialized class of white blood cells called polymorphonuclear neutrophils. These cells normally circulate in the blood stream and, upon activation, adhere to and squeeze through the vascular endothelium, crawling to the infection and inflammation. There they phagocytose bacteria (Figure 4.1) and release a number of proteases with antimicrobial activity [1].

Figure 4.1 A primary neutrophil chasing bacterium S. aureus.
4.2. Signaling mechanisms underlying neutrophil polarity and chemotaxis

Chemotaxis is a fundamental biological phenomenon that is observed both in prokaryotic cells as well as eukaryotic mammalian cells. However the ability to sense the gradient of chemoattractants is controlled by different mechanisms in different cell types. Bacterial cells use a simple trial-and-error approach to translocate up the gradient. The higher mammalian cells show ability to translocate even with shallow gradient by amplifying small external differences in chemoattractant concentration into higher intracellular biochemical gradient. This is achieved by sophisticated mechanisms that exist inside the cells. Neutrophils are good model systems to study the mechanisms involved in chemotaxis.

For neutrophils to chemotax even in the absence of gradients, cells have to be polarized at the leading edge by the formation of F-actin-rich lamellipodia. In the presence of chemoattractants cell polarization is stabilized by stimulation of F-actin polymerization. When there is a chemoattractant gradient, cells can detect gradient and polarize in the direction of gradient. When there is a chemoattractant gradient, cells detect the gradient, polarize and migrate in the direction of gradient. A polarized cell is characterized by its elongated shape and blunt leading edge and narrow posterior. Distinctive actin-myosin contractile complexes are also formed at the sides and back of the cells (Figure 4.2). There are many other regulatory molecules apart from F-actin and actomyosin that show polarized distribution. Neutrophil polarization to chemoattractant stimulation represents a striking example of symmetry breaking from an unpolarized state to a polarized one.
Figure 4.2 Polarization of a neutrophil in response to gradient of chemoattractant. Nomarski images of unpolarized neutrophil responding to a micropipette containing the chemoattractant fMLP (white circle) at (a) 5 s, (b) 30 s, (c) 81 s and (d) 129 s of stimulation. Scale bar: 5 μm. Human neutrophils stimulated with fMLP exhibit highly polarized morphology and asymmetric cytoskeletal assemblies [2]. Actin-myosin recruitment in cells treated with a uniform concentration of attractant fMLP for 2 min, fixed and stained. Fluorescence microscopy reveals (e) F-actin (red) in the pseudopod and RhoA (green) at the rear (Reproduced with permission).

4.2.1. Signaling pathways controlling the protrusive edge

In response to external chemoattractant signals, neutrophils develop a protrusion called pseudopod in the direction of the chemoattractant source. This is achieved by the transmission of signal by the receptors on the cell surface to the interior of the cell. To interpret the gradient of the chemoattractant, the cell must identify the portion of its surface that receives the greatest external signal. This involves comparison of signals received at the entire cell surface. The region with the highest signaling level determines the leading edge. This mechanism is known as “compass” mechanism due to its ability to direct actin polymerization to the leading edge of protruding neutrophils [3-5]. The final component of chemotaxis is the stimulation of the regulatory molecules involved in actin polymerization, which serve as readouts for the compass mechanism.
Asymmetric accumulation of signals

During chemotaxis, the chemoattractants first stimulate G protein-couples receptors (GPCRs). These receptors then activate a trimeric G protein. As a result Gαi in neutrophils or Gα2 in *D. discoideum* are inhibited and Gβγ heterodimer is released [6,7]. Gi trimeric protein is essential to mediate chemotactic signal. When the cells are treated with pertussis toxin (PTX) receptor-dependent activation of Gi proteins is prevented, resulting in elimination of pseudopod formation and other leading edge activities involved in neutrophil chemotaxis. Among Gi proteins, Gαi subunit does not directly influence chemotaxis [8]. It is mainly involved in terminating the activity of Gβγ, which seems to regulate signaling pathways involved in chemotaxis [3,5,9]. It has been experimentally demonstrated that inactivation of Gβγ or their downstream effectors impairs neutrophil migration. The downstream effectors include: phosphatidylinositol 3-kinases (PI3Ks), guanosine triphosphatases of the Rho family (Rho GTPases), protein kinase C ζ (PKCζ), cytosolic tyrosine kinases and cytosolic 6 phospholipase A2 (cPLA2) [3]. In a knock-out study of Gβγ protein in *D. discoideum* it was found that the cells did not show chemotaxis, which demonstrated the importance of Gβγ protein in regulation of chemotaxis [10,11].

To understand the intracellular pathways and protein-protein interactions during chemotaxis of neutrophils, fluorescence techniques have been emerged as powerful tools. Particularly, fluorescent proteins (FPs) and Fluorescence Resonance Energy Transfer (FRET)-based biosensors have been successfully utilized to analyze spatial signals in living cells [12]. These probes can be incorporated only into the genetically manipulatable cell lines. One of such model cell lines for neutrophils is Human
Leukemia cell line (HL-60). HL-60 cells are neutrophilic precursors. On differentiation with DMSO, HL-60 cells behave like neutrophils in terms of polarization and migration in the presence of chemoattractants.[13,14]. HL-60 cells lines can be cultured for long term and can be easily manipulated. The advantages of HL-60 cells have made it possible to express dominant proteins, fluorescent probes, small interfering (si)RNAs and small hairpin (sh)RNAs to investigate intracellular mechanisms affecting polarity and chemotaxis [13-20].

Our understanding of mechanisms involved in spatial accumulation of lipids and proteins during chemotaxis has improved by the incorporation of fluorescent probes in neutrophil-like cell lines. By using these fluorescent probes it was found that the cells can precisely detect changing external gradients through some uniformly distributed chemoattractant receptors (complement factor C5a) at the top of the signal transduction cascade [21]. These cells sense gradients by detecting the number of ligand-bound receptors on their surface and then migrate in the direction where their number is greatest [22]. Similar uniform distribution of chemoattractant receptors was observed in the cell surface of *D. discoideum* undergoing chemotaxis [23]. However, it was found that some chemoattractant receptors like CCR5 are asymmetrically localized at the protruding edge of neutrophils [24]. At the bottom of the signal transduction cascade, external gradient causes accumulation of actin and strong polarization of the regulators of actin such as Arp2/3 complex [2].

Even though at the top of the signaling cascade there are some chemoattractants that do not have spatial asymmetry, there are several signaling molecules in the middle of the signaling cascade that exhibit strong asymmetries in response to chemotactic
gradient. This helps to convert relatively shallow gradients signals of chemoattractant to strongly polarized internal responses. Some of the signaling molecules that exhibit strong asymmetry include the lipid product of PI3Ks phosphatidylinositol 3,4,5-trisphosphate (PI[3,4,5]P3) and active (i.e., GTP-bound) Rac (one of the Rho GTPases). To understand the role of these molecules in the signaling cascade, Green fluorescence protein (GFP)-tagged PH domain of the protein kinase Akt/PKB (GFP-PH-AKT) is used as probe for PI(3,4,5)P3 and yellow fluorescence protein (YFP)-tagged p21-binding domain (PBD) of p21-activated kinase (PAK) (YFP-PAKPBD) is used as probe for active Rac. These probes are localized at the protruding edge of cells in the presence of chemoattractants (Figure 4.3) [13,15]. Asymmetric distribution of PIP3 at the cell surface was found to be likely due to the spatial distribution of PI3K. GFP-tagged p110 subunit of PI3Kγ was shown to be enriched at the leading edge of dHL-60 cells [24]. It was also found in human neutrophils that FRET-based biosensor, a probe for active Rac accumulated at the leading edge in the presence of chemoattractants. Similar observations were recorded for GFP-PH-AKT enrichment in mouse neutrophils, and asymmetric lipid distribution in chemotaxing D. discoideum and fibroblasts. This indicates PI(3,4,5)P3 is one of the most significant molecules that shows spatial asymmetry during chemotaxis.
Figure 4.3 PI(3,4,5)P3 exhibits a polarized distribution during chemotaxis. GFP-PH-AKT was used as a probe for the PI3K lipid product PI(3,4,5)P3. (A) The probe is uniformly distributed inside the cytosol of unstimulated dHL-60 cells, but accumulates on the up gradient face of cells exposed to a chemoattractant gradient, delivered by a micropipette containing fMLP as shown in I, II and III. The white asterisk denotes the position of the micropipette. Scale bar: 10 μm. [13]. (B) 3T3 fibroblasts exposed to a gradient of PDGF (white asterisk). Scale bar: 20 μm. [25] (C) D.discoideum exposed to gradient of cAMP (white asterisk). The arrow heads point to the site of GFP-PH-AKT accumulation [26]. Scale bar: 10 μm. (Reproduced with permission).

**The role of PI(3,4,5)P3 in neutrophil chemotaxis**

Enrichment of PIP3 at the protruding edge appears to be an important event that regulates the activation of key signaling molecules involved in neutrophil polarization and migration. Various experimental studies in neutrophils and D. discoideum support this hypothesis. Genetic perturbation of PI3K isozymes lead to defective chemotactic response *in vitro* and diminished recruitment at the inflammation locations *in vivo* [27-29]. Deficiency of PI3Kγ results in decreased production of PIP3 when cells are stimulated with various chemokines such as fMLP, IL8 or C5a. This indicates that the
role of PI3Kγ in the production of lipid products. Pharmacological reduction of PI3K activities with the aid of global PI3K inhibitors such as wortmannin and LY294002 also resulted in reduced leading edge activities and chemotactic response. Different isoforms of PI3K have variation in effects on neutrophil migration in human, mouse as well as in *D. discoideum* [30-33].

Studies involving deletion of PIP3-degrading enzymes showed that enrichment of PIP3 in an appropriate spatial asymmetry is essential for chemotaxis. Deletion of PTEN in *D. discoideum* affected spatial distribution of PH domain and actin polymerization resulting in cells exhibiting multiple leading edges. Consequently, the cells failed to migrate to the chemoattractant source. [34,35]. In *D. discoideum* PI3Ks and PTEN coordinate regulation of spatial enrichment of PIP3 and thereby play a key role in determining cell polarization and migration. [34,35].

The role of PTEN in neutrophil chemotaxis has been subject to debate. In 2002, Hannigan et al. [36], found that lack of PTEN in mouse neutrophils disrupted their directional response, where as recent studies reported that PTEN is not essential for polarity and migration of mouse neutrophils in presence of chemoattractants [37]. Because of such differences in these findings, another PIP3 inhibitor, SHIP1 has been studied [37]. Absence of SHIP1 in neutrophils had lead to the development of increased number of membrane protrusions. As a result, these cells lack polarization and show defective migration.

Even though PIP3 distribution and regulation has been shown to be a key component of cell’s compass, it may not be the only factor regulating cell chemotaxis [27-29,37-40]. It appears that there exists a parallel pathway that influences cell’s
compass [39]. Thus there are two redundant pathways controlling the chemotaxis in neutrophils and *D. discoideum*, where one is dependent on PIP3 and the other is independent.

Many studies have been conducted to understand how the shallow external gradient signal is converted to steeper internal signal [41-44]. This has been explained by a model that suggests two competing processes with different spatial characteristics determine cell polarization. Experimental studies show that PIP3 and Rac act as signals in positive feedback look and are responsible for amplification of external signal and leading edge activities of neutrophils [13-15,45-47]. To get deeper insight into the molecular mechanisms governing feedback loops, further investigation needs to be performed.

### 4.2.2. Backness signaling pathways

During chemotaxis neutrophils require to pull their trailing edges, called as uropods, after extending the protruding edge for maintaining polarity and motility. This mechanism cannot be explained by the frontness pathway itself. Neutrophil polarization and migration under uniform stimulation conditions also cannot be explained by frontness pathway alone. In recent studies an alternative “backness” pathway has been suggested to explain contraction and de-adhesion of the uropods of cells [46].

Experiments performed with PTX support hypothesis of backness signaling pathway. PTX blocks polymerization of actin, leading edge formation, PIP3 enrichment and activation of Rac [13,46]. Cells that are treated with PTX responded to chemoattractant signal in a characteristic way. These cells develop a well-defined uropod-like structure instead of a pseudopod as in the normal cells. This suggests that a separate pathway
is involved in determining morphological backness of a neutrophil exposed to chemoattractants [46,48]. This pathway is independent of Gi and is initiated by trimeric G proteins G12 and G13 along with the downstream elements including PRG (PDZRhoGEF, a Rho specific GEF), Rho, p160-ROCK (a Rhodependent kinase), phosphorylated myosin light chain (p-MLC), and consequent activation of myosin II [20,46]. A key feature of backness is inhibition of frontness promoting effects of the chemoattractant. On the other hand, blocking the backness pathway makes the entire cell sensitive to the chemoattractant.

Chemoattractant signals can effectively activate formation of distinct actin assemblies in neutrophils. A model has been proposed based on these effects to elucidate self-organizing polarity and asymmetric attractant sensitivity. This model comprises of self-organization of actin cytoskeleton, where actin polymer assemblies forms in the front, while actin myosin assemblies form at the back (Figure 4.4). Such formation of assemblies plays an important role in transmission and modulation of intracellular signals. This model can explain polarization of neutrophils in uniform chemoattractants. It can also explain why neutrophils respond to changes in direction of a gradient by performin U-turns rather than simply reversing polarity [46,49].
Figure 4.4 Distinct actin assemblies modulate sensitivity to attractant and self-organizing polarity of neutrophils. Chemoattractant binds to a GPCR (R), which in turn activates different trimeric G proteins to generate two divergent, opposing signaling pathways, which promote actin polymerization (frontness) and actin-myosin contraction (backness), respectively. Localized mechnochemical incompatibility of the two cytoskeletal responses, combined with the ability of each to damp signals that promote the other (dashed inhibitor lines), then gradually drive them to separate into distinct domains of the membrane. As a result, a morphologically distinct pseudopod, which is highly sensitive to attractant, demarcates itself from relatively insensitive membrane, enriched with myosin, at the back and sides [50].

4.3. Neutrophil behavior in complex array of gradients

Neutrophils are uniquely sensitive to a wide variety of chemoattractant signals emanating from endothelial cells lining the capillary wall, activated cells near sites of inflammation or sources or information. These chemoattractants include: (i) formylmethionylleucylphenylalanine (fMLP) secreted by the infecting microbes; (ii) chemokines such as interleukin-8 (IL-8), growth-related gene product α (GROα), leukotriene B4 (LTB4), and stromal cell-derived factor 1 (SDF-1) secreted by damaged tissue, mast cells, monocytes, and also by neutrophils themselves; and (iii) a glycoprotein fragment, C5a, produced by the complement system [51-56]. Neutrophils migrate in the presence of any of these chemoattractants. While approaching their
targets, neutrophils are encountered with a complicated network of signals coming from multiple chemoattractant sources (Figure 4.5). Some signals are caused due to infection while others are generated by damaged tissues. During chemotaxis, neutrophils use a kind of logic to prioritize and distinguish the signals that they receive [57-59].

Figure 4.5 Neutrophils encounter a complex array of chemoattractant signals while approaching their targets.

4.3.1. Signaling hierarchy

Neutrophil migration from bloodstream to the site of infection is a fascinating phenomenon. Little is known about how neutrophils are able to precisely find their target in response to such a complex network of pro-inflammatory signaling molecules (Figure 4.6). Many studies have been conducted to understand the response of neutrophils against multiple chemoattractant sources. A preliminary hypothesis
suggests that neutrophils are unable to distinguish between the chemoattractants and they move towards the vector sum of sensed gradients.

Figure 4.6 How does neutrophil choose between two opposing chemoattractant gradients (denoted by the blue and red circles)?

For example when neutrophils are exposed to two gradients produced by chemoattractants, IL-8 and LTB4, the neutrophils migrate as if exposed to a single chemoattractant without differentiating between the two chemoattractant gradients. Using under agarose assays, Foxman et al. [58] observed successful integration of directional signals from chemoattractant sources presented at an angle to neutrophils. In the presence of a single chemoattractant IL8 or LTB4, cells migrated toward that source. However, in the presence of two different chemoattractant sources, IL8 and LTB4 in each well, cells migrated in a broad front, with the majority of cells migrating furthest in an intermediate direction between the two sources, suggesting vector integration of signals (Figure 4.7).
Integration of directional signals from chemoattractant sources. A neutrophil-containing well and two chemoattractant source wells were placed in an equilateral triangle. Chemoattractant source wells contained medium, IL-8 (10 pmol), or LTB4 (10 pmol). Cells were allowed to migrate for 150 min, after which they were fixed, stained, and photographed [58].

In many experiments it was determined that fMLP and C5a are always preferred by neutrophils over IL-8 or LTB4. This behavior of neutrophils preferring a particular chemoattractant over the other would breakdown the vector sum hypothesis. It was observed that neutrophils are first migrated to saturated concentrations of one chemoattractant before translocating to second chemoattractant. In the presence of fMLP or C5a, neutrophils ignore the signals produced by other sources (Figure 4.8). The data supports a model of preferential selection of chemoattractants by the neutrophils. It was also found that one chemoattractant may enhance the sensitivity to another chemoattractant. For example, SDF-1 and monocyte chemotactic protein 1 (MCP1) increases effective migration of neutrophils in IL-8 gradients in proportion to the concentration of chemokines. Depending on the context and order in which neutrophils sense chemoattractants, the response to chemoattractants is additive, synergistic, or antagonistic.
Figure 4.8 Chemotactic behavior of neutrophils originating at a source of fMLP. (a) Photographs of stained cells after 2 hr migration to a source of fMLP, IL-8, or LTB4 (1 pmol). Cells originating in an agarose well containing IL-8 or LTB4 (10 pmol) exhibit robust migration towards fMLP (top row); however, cells placed with fMLP (10 pmol) do not migrate towards IL-8 or LTB4 [57].

4.3.2. Mechanisms of selective migration

A number of mechanisms have been proposed explaining preferential migration of neutrophils in the presence of multiple chemoattractants. One of the mechanisms, based on receptor cross-desensitization, proposes that the receptors of the neutrophils for one chemoattractant are desensitized because of the binding of another chemoattractant. It has been observed that the receptors for IL-8 subtype CXCR2 on cell surface are inhibited by fMLP and C5a [60]. On the other hand, some studies have found that receptors for fMLP were increased while the receptors for IL-8 were decreased because of the effects of IL-8 [61,62]. The ability of cells to preferentially select distant source can be explained in terms of their sensitivity to chemoattractants. Sensitivity is a function of ligand concentration. Neutrophils show an optimal concentration range for each chemoattractant in which they are most sensitive [63] (Figure 4.9).
Figure 4.9 Cell orientation as a function of concentration of FMMM. The orientation of cells exposed to a 10-fold (upper curve) or threefold (lower curve) concentration gradient across the bridge was scored. The concentrations on abscissa indicate the high concentration of FMMM present in the gradient being scored. The results are presented as the mean percentage of cells oriented plus or minus the SEM (n= 5, 11, 10, 4, 8, and 2 for the tests of increasing concentrations in upper curve, and n= 4, 3, 4, 3, 4, 3 for the tests of the lower curve) [63].

According to another mechanism, different pathways govern regulation of chemoattractants. A two pathway signaling model has been proposed in which the end target chemoattractants activate p38 mitogen-activated protein kinase (MAPK) that inhibits intermediary chemoattractant-induced PI3K/Akt pathway [59]. Another mechanism suggests a peculiar biphasic response of neutrophils to chemoattractants [64]. Neutrophils have been shown to migrate towards or repel against IL-8 depending on the concentration gradients of IL-8.

A recent study has also identified PTEN as a necessary component for neutrophils to prioritize and integrate responses to multiple chemotactic cues (Figure 4.10) [65], but a more detailed dissection of the molecular mechanisms is still lacking. Molecular
insights into this puzzle should prove valuable in understanding neutrophils navigation in complex environments and may yield important information for inflammation and even cancer metastasis.

Figure 4.10 Effect of PTEN deficiency on chemotaxis to a single gradient of fMLP or CXCL2. Data are presented as mean and s.e.m.; *, P < 0.05, compared with wild type (Students t-test)[65] (Reproduced with permission).

4.3.3. Regulation of chemokine production

Neutrophils play an active role in the regulation of production of inflammatory chemoattractants [66]. When neutrophils are exposed to LPS, they produce cytokines, interleukin 1 β (IL-1β) and tumor necrosis factor α (TNF-α) [67]. By the influence of these cytokines, production of chemokines such as IL-8 is increased as well as neutrophils response to IL-8 is enhanced. However when neutrophils are exposed to both LPS and IL-10, IL-1 receptor antagonist (IL-1ra) is generated. IL-1ra causes the blockage of the IL-1 effect [68]. In response to different stimuli, neutrophils produce chemoattractants in addition to the generation of cytokines [66]. Some of the chemoattractants are specific for immune cells involved in later stages of inflammatory response such as macrophage inflammatory protein (MIP) 1α and 1β. Other
chemoattractants such as IL-8 and GROα, and LTB4 are specific for neutrophils. Of these chemoattractants IL-8 stimulates its own production.

Chemokine production in an autocrine or paracrine manner may be attributed to its role in serving as a feedback mechanism for controlling the number of cells at the site of inflammation. Alternatively, cell-generated chemoattractants may play a role to either immobilize cells or influence their response to other chemoattractants through receptor crosstalk. In either case, it is evident that neutrophils take an active part in regulation of innate immune response.

4.4. Scope of future research

In response to inflammation, neutrophils migrate using a directed path through a complex network of signals produced by multiple chemoattractant sources. Migration of cells under the influence of a single chemoattractant has been well studied. However, migration of cells in presence of multiple chemoattractants is not completely understood. Also, the mechanism by which immune system drives neutrophils towards the target sites using various cues is not adequately understood. Neutrophil navigation is a complex process that involves coupled regulation of inter and intracellular signaling networks spanning multiple scales in both time and space. The decision to follow chemotactic cues is determined by single cells, while the production and evolution of these cues is determined by the spatial distribution of and interactions between these cells and their environment. A combination of novel mathematical and experimental techniques that target different spatiotemporal scales is required to explicate the regulatory mechanisms involved in neutrophil chemotaxis.
4.5. References


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Chapter 5 Microfluidic platforms for neutrophil chemotaxis

5.1. Introduction

Gradients of biomolecules, such as chemokines and growth factors play a key role in guiding the growth, migration, and differentiation of cells within the 3D in vivo environments. A growing interest in elucidation of these phenomena has motivated the development of various in vitro methods to expose cells to chemical gradients.

5.2. Conventional chemotaxis platforms

Conventionally, several lab scale gradient generating platforms have been developed to mimic in vivo gradients. One of the earliest methods include biological hydrogels [1-4] formed in couple of the ways. The first approach is to co-culture cells with another cellular source capable of releasing the desired biomolecule [2]. Another approach is to make wells within the gel (Figure 5.1) and fill them with the soluble form of the biomolecule at a known concentration [3]. Biological hydrogels are easy to make and provide an environment to cells, that is very similar to one found within a living tissue. High network density of gels allows the movement of chemical species to occur only via free diffusion.

Figure 5.1 A neural tissue explant co-cultured with semaphorinexpressing COS cells in a collagen gel [2], and (b) Neutrophils (black dots) migrating in overlapping gradients of IL-8 and leukotriene LTB4 in an under-agarose assay [3] (Reproduced with permission).
Boyden developed a gradient-generating assay, based on a chamber of two medium-filled compartments separated by a microporous membrane [5]. The lower compartment comprises of a chemoattractant solution. The chemical gradient results by diffusion of chemoattractant from the lower to the upper compartment through the membrane (Figure 5.2). Cells are placed in the upper compartment and are allowed to migrate through the transmembrane pores to the lower compartment. After an appropriate incubation time, the membrane between the two compartments is fixed and stained, and the number of cells that have migrated from upper compartment to the lower one is determined. The assay is easy to perform and gives a quantitative measure of the level of migration of cells across the membrane.

![Figure 5.2 Schematic of Boyden chamber assay](Reproduced by permission of The Royal Society of Chemistry)

Zigmond chamber was the first assay developed to directly visualize cell behavior in chemoattractant gradients (Figure 5.3). The device comprises of two etched channels separated by a glass ridge. Cells are seeded on a glass coverslip and the coverslip is inverted over the etched channels and their migration in response to the gradient established between the coverslip and the glass ridge is recorded. Dunn chamber is also based on similar principle as Zigmond camber, but is much less susceptible to
evaporative losses. Therefore, it can be used for studying long-term chemotaxis, particularly in slowly moving cultured cells such as fibroblasts.

Figure 5.3 (a) Zigmond chamber, and (b) a schematic showing principle of cell migration in Zigmond chamber [6] (Reproduced by permission of The Royal Society of Chemistry).

Another gradient-generating method that has been more recently been developed is through the use of drawn glass micropipettes (Figure 5.4). The micropipette is filled with a chemoattractant and its tip is positioned to a set distance from cells. Solution is either allowed to passively diffuse from the tip or is pneumatically ejected out of the pipette into the extracellular environment. The method is particularly well suited for characterizing single cell responses and can easily be extended to generate combinatorial gradients by using multiple micropipettes.

Figure 5.4 Micropipette filled with chemoattractant solution are mounted in micromanipulators arranged around the cell culture dish [6] (Reproduced by permission of The Royal Society of Chemistry).
The conventional methods have contributed significantly in the identification of new biomolecules that bring forth gradient-dependent cell responses and unraveling various aspects of mechanisms involved in the process. Chemotaxis phenomena has so far been understood to be a complex, highly-regulated process, involving multiple signaling pathways, that integrates the intracellular signals generated by all the chemical gradients to which a cell is exposed. However, further illustration of chemotaxis is limited by lack of quantitative knowledge about nature of gradients. The aforementioned classical gradient-generating methods produce temporally and spatially varying gradients. Poorly defined absolute concentrations and gradient profiles make it difficult to determine the optimum conditions for cell response. These methods produce unstable and non-linear gradients that cannot be easily reproduced. Most of these platforms are unsuitable for generating multiple independent gradients. Collectively, these limitations make it very difficult to correlate specific cell responses with gradient characteristics.

5.3. Microfluidic gradient platforms

Majority of the issues faced in classical gradient-generating platforms have been addressed using microfluidic-based methods (Figure 5.5). Microfluidic gradient generators can produce stable and reproducible gradients that can be easily quantified. Most of the methods allow precise spatial and temporal control of gradient conditions. The ability of create user-defined and controllable gradient microenvironments make these devices powerful tools to understand gradient signaling processes in cells [7]. There are additional practical advantages of high throughput, low cost, requirement of
small reagent volumes, adaptability to diverse cell types, and application-dependent modification in design of device [6].

Microfluidic gradient platforms have been successfully demonstrated for a wide variety of applications, such as to infect cells with graded concentrations of virus [8], produce gradients of varying stiffness [9] and cell-adhesion molecules in synthetic extracellular matrices [10], create adsorbed gradients of extracellular matrix proteins [11-13], induce stem cell proliferation and differentiation [14], and examine the effects of various chemoattractant gradients on chemotaxis of neutrophils [15-18], bacteria
[19,20], sperm[21], breast cancer cells [22,23], and intestinal cells [13]. Advances in microfabrication techniques and improved understanding of fluid flows at the micrometer scale have lead to design and development of microfluidic platforms tailored for specific applications. The devices can be broadly classified as flow-based gradient platforms [15,17,19,24-31] and diffusion-based gradient platforms [20,32-39].

Here we use flow-based gradient generating platforms to investigate neutrophil chemotaxis. Using principle of laminar flow and controlled diffusive mixing, these microfluidic platforms allow formation of stable concentration gradients (Figure 5.6). Flow is continuously driven by syringe pumps and gradients can be maintained indefinitely.

![Diagram](image.png)

**Figure 5.6** Laminar flow-based microfluidic platforms for neutrophil chemotaxis. (a) “Christmas-tree” network based device [15] (Reproduced with permission), and (b) a simple Y-channel device.
5.4. Materials and methods

5.4.1. Cell culture

Differentiated HL-60 (Human promyelocytic leukemia) cells are used here as a model for the study of neutrophils [42-45]. HL-60 cells are predominantly neutrophilic promyelocytes. On differentiation, they exhibit neutrophil morphologies, polarize in response to chemoattractants, and migrate in gradients of chemoattractant at rates comparable to primary neutrophils [46-48]. HL-60 cells were purchased from ATCC and cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin G. Cells were periodically subcultured to remain in the subconfluent condition (0.6 x 10^6 to 1.0 x 10^6 cells/ml). For differentiation into neutrophil-like cells, HL-60 cells were diluted to 0.1x10^6 cells/ml in fresh growing medium containing 1.3% DMSO (final concentration). Cells were propagated for 6 or 7 days without changing the medium [47,49].

5.4.2. Preparation of chemoattractant solution

Solutions of chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP) were prepared by diluting in Hank’s Buffered Salt Solution (HBSS). Fluorescent indicators were added to the solution in order to visualize the gradients.

5.4.3. Microfluidic device fabrication

The microfluidic gradient device consists of a poly (dimethylsiloxane) (PDMS) slab embedded with microfluidic channels and glass substrate as its base. The microchannels were fabricated standard photolithographic methods as described previously [41,50,51]. Briefly, channel design is printed on a transparency film using a high-resolution printer. The photomask was used to fabricate 50 µm high SU-8
photoresist features on a Silicon wafer using UV-exposure lithography. PDMS pre-polymer was then poured on Silicon master and allowed to cure for 2 hours at 65 °C. The PDMS replica was then peeled off the Silicon master. Flow inlets and outlets were made in PDMS using a steel punch. The surface of the PDMS replica and a clean glass coverslip (Fisher Scientific) were exposed to plasma atmosphere for 90 s (Model PDC-001, Harrick Scientific) and irreversibly bonded together to complete the device assembly. The device was connected to a syringe pump (Harvard Apparatus) with PTFE tubings (Cole-Parmer) and 1ml syringes (BD Biosciences) via 23 G 3/4 size needles (BD Biosciences) in order to deliver fluid and cells to the microchannels. Fibronectin or Fibrinogen (250 µg/ml) was coated inside the microfluidic device for 1 hour at room temperature before performing any chemotaxis experiment. A new microfluidic device was used for each chemotaxis experiment.

5.4.4. Microfluidic gradient generation

Microfluidically generated gradients were visualized using fluorescence microscopy. Syringe pumps were used to automatically infuse fluorescently labeled solution from one inlet and an unlabeled solution from another inlet of the microfluidic device. The gradient is quickly established by controlled diffusive mixing across the interface of laminar flow streams. Fluorescent images were captured at different locations along the length and the width of the channel using FITC filter on a Zeiss Axiovert 200M microscope (Figure 5.7). Image analysis was performed using ImageJ. Fluorescence intensity profiles validate the formation of a well-defined, linear and stable solution concentration gradient as demonstrated previously [41].
Figure 5.7 Gradient visualization using fluorescence microscopy. Fluorescent images acquired at the (a) junction of individual branches and the main channel in a Christmas-tree network device, and at the (b) junction of two inlets and main channel in a Y-channel device. Laminar flow quickly establishes a smooth well-defined gradient down the channel as seen in (c).

5.4.5. Microfluidic chemotaxis assays

Cells were washed and suspended in modified Hank’s balanced salt solution (mHBSS) containing 1% Human Serum Albumin (HSA) and injected in the microfluidic device. The device was left in the incubator (37°C, 5% CO2) for 20 min to allow the cells to adhere to the substrate. The channels were then gently rinsed with mHBSS to wash away floating cells. Chemoattractant fMLP and mHBSS solutions were allowed to flow into the device from separate inlets at flow rate of 0.02 ml/hr to generate soluble concentration gradients and cell response was observed and recorded.
5.4.6. Time lapse microscopy

Differential interference contrast (DIC) images and fluorescent images were captured with a Zeiss 40X NA 1.30 Fluar DIC objective on a Zeiss Axiovert 200M microscope. All images were acquired with a cooled charge-coupled device camera (AxioCam MR3, Zeiss), at an interval of 10 s for the duration of the experiment (~20 minutes).

5.5. Cell response under different gradient conditions

Firstly, the conditions for cell migration in microfluidic chambers were successfully optimized. Initial issues with improper cell adhesion were addressed by varying the type of protein coating (Fibrinogen, Fibrinectin and BSA), concentration of protein coating and incubation times. The migration of dHL-60 cells in uniform concentration conditions of fMLP and MHBSS served as control experiments. Each of these showed random motility in agreement with previously reported diffusion-based and microfluidic assays [16,52]. Figure 5.8 shows time lapse images of dHL-60 cells migrating in a microfluidically generated gradient device.
Figure 5.8 Cell migration in microfluidic device. Optical micrographs show cells at (a) t=0, (b) t=7 minutes, and (c) t=15 minutes, in a 0-500 nM fMLP gradient.

Next, the chemotactic migration of dHL-60 cells in linear fMLP gradients was assessed across a 500 μm wide microchannel. Five linear fMLP gradients were tested, including (a) 0–25 nM (slope=0.07 nM/μm), (b) 0-50 nM (slope=0.14 nM/μm), (c) 0-100 nM (slope=0.28 nM/μm), (d) 0-250 nM (slope=0.7 nM/μm), and (e) 0-500 nM
(slope=1.4nM/μm). The experiments show that cells stop migrating up the gradient after a certain distance (Figure 5.9a-c).

Figure 5.9 Cell saturation in microfluidic gradients. Optical micrographs show cells in fMLP gradient of slope (a) 0-50 nM, (b) 0-100 nM, and (c) 0-250 nM. All time lapse images were acquired at the end of migration experiment.

The saturation distance was observed to increase on decreasing the slope of gradient from 0-500 nM to 0-50 nM (Figure 5.10). Although in case of 0-25 nM gradient, not all cells at the bottom of gradient seemed to respond, so saturation phenomenon
was not distinctly observed. However, the absolute local concentration values at which cell were observed to reach saturation was not same in different gradient conditions. This suggests that both local concentration and slope of the gradient are crucial in determining cell saturation. The observed phenomena is hypothesized to be governed by two factors: 1) how many signaling molecules does a cell see around, and 2) when do chemokine receptors on surface of cell reach saturation limit.

![Graphs showing relationship between concentration gradient and cell saturation](image)

Figure 5.10 (a) Distance at which cells reach saturation and become insensitive to show any further directional movement up the gradient. (b) Absolute local concentration at saturation point. All distances were measured from the bottom edge of channel (low concentration) along the width of the channel.

5.6. Conclusions

The microfluidic platforms were successfully demonstrated to generate stable chemoattractant gradients. Chemotaxis experiments were performed using dHL-60 cells in fMLP gradients under different gradient conditions. The observations suggest a clear influence of both local concentration and slope of gradient on cell migration. The hypothesis needs to be explored by developing a modeling framework for chemotaxis in microfluidic platforms and comparing experimental results with model predictions (Keller Segel based models). Future experiments should aim at further investigation of cell
saturation phenomenon using other chemoattractants under different gradient conditions. However, this requires use of primary neutrophils since receptors for some of the neutrophil chemoattractants are absent on dHL-60 cells. The exhaustive and systematic analysis of cell migration under various conditions will identify the correlation among cell saturation, gradient characteristics, and the chemoattractant used.

5.7. References


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Chapter 6 Protein localization during neutrophil chemotaxis

6.1. Introduction

Biomolecular gradients play a key role in regulating many biological/physiological phenomena, such as immune defense, tissue regeneration, morphogenesis, tumor metastasis, and axon guidance [1-3]. An increasing interest to unravel these complex biological processes has led to the development of various techniques to replicate gradient conditions found in vivo. Conventionally, various in vitro gradient-generating methods have been employed for studying chemotaxis and axon guidance, including under-agarose assay [4-6], Dunn chamber [7], Zigmond chamber [8], Boyden chamber [9], and more recently micropipette assay [10-12]. These platforms have played a significant role in shaping our understanding of the phenomena underlying directed growth, differentiation and migration of various cell types [4,13-17]. However, most of our understanding has been limited to being qualitative in nature due to inherent limitations of these platforms. The chemical gradients generated using these methods often show spatiotemporal variation, and cannot be easily controlled and reproduced. Most of these methods are also limited in their ability to generate multiple, independent gradients.

Advances in microfluidic and microfabrication technology provide a promising alternative in form of laminar flow based chemotaxis platforms capable of producing predictable, stable and reproducible gradients. The gradient profiles can be mathematically quantified and therefore precisely controlled over space and time [18,19]. The use of microfluidic gradient platforms has been successfully demonstrated
for investigation of neutrophil chemotaxis as well as other biological applications [20-25]. Agrawal et al. [26] demonstrated isolation of neutrophils on a chip directly from whole blood by using selectin-modified substrates. Lin et al. [21] used these devices to investigate role of mean chemoattractant concentration and gradient steepness on effective neutrophil chemotaxis. The devices have also been extended to study neutrophil response in competing gradients of chemoattractants [25]. The platforms can be tailored to probe into complex mechanisms underlying neutrophil chemotaxis. Irimia et al. [27] demonstrated a device with a microstructured membrane that can allow investigation of neutrophil migratory response to fast and precise gradient switches between stable gradient conditions. Tharp et al. [28] demonstrated persistent, directionally biased movement away from microfluidically generated IL-8 gradients, suggesting the role of chemorepulsion in the homeostatic control of inflammation. Here we employ these microfluidic gradient platforms to investigate recruitment of key regulatory molecules in response to different gradient conditions during neutrophil chemotaxis.

*Neutrophil polarization*

Neutrophil chemotaxis is a complex phenomenon characterized by morphological polarization and migration of cells upstream of concentration gradients of chemoattractants. In view of the critical physiological functions of neutrophils, their chemoattractants and chemoattractant receptors targets have remained the main areas of investigation [29]. Much less is known about mechanisms involved in neutrophil polarity and directional migration [30]. Chemotaxis involves chemoattractant-induced selective localization of number of regulatory protein and lipid signaling molecules to
either the front or back of the polarized neutrophils [31]. Filamentous actin (F-actin) is polymerized asymmetrically at the upgradient edge of the cells. Characteristic actin-myosin contractile complexes are formed at the sides and back of the cells. Spatial localization of these molecules is responsible for determining cell orientation and direction of migration. Different approaches can be taken to explore the roles of a number of different signaling molecules in the chemotactic pathway. One can perturb a signaling pathway by adding poisons selective for different proteins or by knocking out specific genes using dominant negatives or siRNA. These tools have been successful in providing evidence to indicate that a number of antagonizing feedback loops control polarization [31,32]. An alternative approach is to use a combination of immunostaining and protein tags to monitor selective recruitment within the cell [11]. With ongoing developments in labeling methods, imaging and analytical techniques, this approach to study receptor localization and function is emerging as a powerful tool to understand intracellular pathways [33]. The method has been successfully used to investigate qualitative aspects of localization of key regulatory molecules in neutrophils and in amoebas of Dictyostelium discoideum (D. discoideum) [11,34-36]. In this work, we establish microfluidic platforms as tools for quantitative analysis of intracellular phenomena involved in chemotaxis. We specifically demonstrate a novel methodology for quantification of localization of regulatory molecules Actin and PHAKT in signaling pathways.

Chemotaxis involves spatially localized rearrangement of cytoskeleton to effect cell migration in appropriate direction. Phosphatidylinositol-3,4,5-triphosphate(PIP3), a membrane-associated lipid product of the phosphatidylinositol-3-OH kinases (PI3Ks) is
shown to strongly localize in chemoattractant gradients. The internal gradient of PIP3 gets amplified in presence of external gradient conditions, resulting in ability of cells to detect and migrate up very shallow gradients [11,37]. PIP3 also promotes the activity of Rac and Cdc42, which in turn, initiate actin polymerization at the leading edge by activating p21-activated kinases (PAK) [32]. Thus, the regulation of PIP3 is of crucial importance in directing recruitment and activation of signaling components involved in cell polarization and chemotaxis. In this work, we specifically want to address whether amplification of internal signal is dependent on exterior conditions. The methodology developed can be extended to investigation of various other involved biomolecules and intracellular processes of significance.

6.2. Materials and methods

6.2.1. Cell culture, differentiation and transfection

HL-60 cells were purchased from ATCC and maintained in RPMI 1640 supplemented with 25 mM HEPES, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin G. Cell culture was routinely split to remain in the subconfluent condition (0.6 x 10^6 to 1.0 x 10^6 cells/ml). For differentiation into neutrophil-like cells, HL-60 cells were diluted to 0.1x10^6 cells/ml density in fresh growing medium containing 1.3% DMSO (final concentration). Cells were propagated for 6 or 7 days without changing the medium [10,38].

HL-60 cells stably expressing actin-YFP or PHAKT-YFP were made in Henry Bourne’s lab (University of California, San Francisco). Briefly, HL-60 cells were harvested at log phase. Cells were washed twice with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} and suspended in 400 µl ice-cold RPMI 1640 supplemented with
25 mM HEPES (2 x 10⁷ cells/ml). Plasmids containing actin-YFP or PHAKT-YFP (30 µg) were added to cell suspension and cells were then electroporated by Gene Pulser II electroporator (Bio-Rad). Transfected cells were selected for 4 to 5 weeks with 1 mg/ml active G-418. HL-60 cells stably expressing actin-YFP or PHAKT-YFP were further concentrated by fluorescence-activated cell sorting (FACS) [11].

6.2.2. Microfluidic device fabrication

The microfluidic gradient device comprises of a poly (dimethylsiloxane) (PDMS) slab embedded with microfluidic channels and glass substrate as its base. The microchannels were fabricated using rapid prototyping and soft lithography as described previously [19,39,40]. Briefly, high resolution printing (5080 dpi) was used to print a photomask with design pattern on a transparency film. The mask was used to fabricate 50 µm high SU-8 photoresist features on a Silicon wafer using photolithography. PDMS molds with embossed channels were fabricated by curing the pre-polymer on Silicon master for 2 hours at 65 °C. The PDMS replica was then peeled off the Silicon master. Inlets and outlets for the fluids and cells were created in PDMS using a steel punch. The surface of the PDMS replica and a clean glass coverslip (Fisher Scientific) were treated with air plasma for 90 s (Model PDC-001, Harrick Scientific) and irreversibly bonded together to complete the device assembly. The device was connected to a syringe pump (Harvard Apparatus) with PTFE tubings (Cole-Parmer) and 1ml syringes (BD Biosciences) via 23 G 3/4 size needles (BD Biosciences) in order to deliver fluid and cells to the microchannels. Fibronectin (250 µg/ml) was coated inside the microfluidic device for 1 hour at room temperature before use in each chemotaxis experiment.
6.2.3. Microfluidic gradient generation

The gradient formation was verified by infusing fluorescently labeled solution from one inlet and an unlabeled solution from another inlet of the microfluidic device. Controlled diffusive mixing across the interface of laminar flow streams quickly establishes the gradient. Fluorescent images were acquired at different locations along the channel using FITC filter on Zeiss Axiovert 200M microscope. ImageJ was used to analyze fluorescent intensity profiles across the channel width. The profiles suggest formation of a well-defined, linear and stable solution concentration gradient as demonstrated previously [19].

6.2.4. Chemotaxis experiments in microfluidic device

Cells were washed and suspended in modified Hanks' balanced salt solution (mHBSS) containing 1% Human Serum Albumin (HSA) and injected in the microfluidic device. The device was kept in the incubator (37°C, 5% CO₂) for 20 min to allow cells to attach to the substrate. Unadhered cells were washed away by gently rinsing the channels with mHBSS. Chemoattractant fMLP and mHBSS solutions were infused into the device from separate inlets at the flow rate of 0.02 ml/hr to establish soluble concentration gradients. Differential interference contrast (DIC) images and fluorescent images were captured with a Zeiss 40X NA 1.30 Fluar DIC objective on a Zeiss Axiovert 200M microscope. All images were collected with a cooled charge-coupled device camera (AxioCam MR3, Zeiss).

6.2.5. Quantitative Analysis

ImageJ was used in conjunction with the Image Processing Toolbox in MATLAB to analyze fluorescent micrographs captured from the microscope. A custom MATLAB
script identified both the point of peak fluorescence in the cell (typically located near the leading edge), as well as the geometric centroid based on the cell outline. These two points were then used to determine the cell orientation relative to the external gradient in the microfluidic channel. In addition, a gaussian weighting function centered on the peak fluorescence was defined with a set standard deviation of 2 µm. This function was used to weight the integral of total normalized fluorescence in the cell around the peak, providing a quantitative measure of protein localization for both Actin and PHAKT.

6.3. Results and discussion

6.3.1. PHAKT and Actin localization

Differentiated HL-60 cells (dHL-60) stably transfected with the pleckstrin homology domain of Akt fused to yellow fluorescent protein(YFP) were used in microfluidic devices in order to investigate localization of PHAKT in response to external gradient conditions. Similarly, dHL-60 cells transfected with Actin-YFP were used to determine Actin localization. Cells were exposed to different gradient conditions. The migration of cells was examined under seven different gradient conditions: (1) 0-10 nM (slope=0.028 nM/µm), (2) 0-25 nM (slope=0.07 nM/µm), (3) 0-50 nM (slope=0.14 nM/µm), (4) 0-100 nM (slope=0.28 nM/µm), (5) 0-125 nM (slope=0.35 nM/µm), (6) 0-250 nM (slope=0.7 nM/µm), and (7) 0-500 nM (slope=1.4 nM/µm).
Figure 6.1 PHAKT-YFP labeled dHL-60 cells in a microfluidic gradient device as visualized through fluorescence microscopy. Time lapse micrographs of a single cell in (a) show PHAKT recruitment at time instants, (b) 10s, (c) 150s, (d) 250s, (e) 350s, (f) 600s, and (g) 1220s after stimulation of cells to fMLP gradient. Steepness of gradient was 0.7 nM/µm. Scale bar: 10 µm.

Time lapse images show distribution of PHAKT across cell length as cell migrates in response to the gradient (Figure 6.1). Initially, cell is unstimulated in the absence of any chemoattractant. It exhibits round morphology and PHAKT is uniformly distributed across the entire cell. When the gradient is applied, cells become polarized and start migrating up the gradient. On reaching a saturation distance, PHAKT again redistributes itself across the cell length. Similar recruitment pattern is obtained in case of Actin-YFP cells (Figure 6.2).
Figure 6.2 Actin-YFP labeled dHL-60 cells in a microfluidic gradient device as visualized through fluorescence microscopy. Time lapse micrographs of a single cell in (a) show Actin recruitment at time instants, (b) 10s, (c) 150s, (d) 250s, (e) 350s, (f) 600s, and (g) 1220s after stimulation of cells to fMLP gradient. Steepness of gradient was 0.7 nM/µm. Scale bar: 10 µm.

A qualitative comparison of Actin and PHAKT recruitment shows a difference in pattern of localization of each protein. Actin is localized at the front edge, while PHAKT is localized at the membrane. A detailed quantitative analysis was performed to investigate effect of external gradient conditions on protein localization. In order to perform the analysis, fluorescence images of individual cells need to be segmented. Automated analysis is limited to conditions when there is no overlap between cell boundaries. However, it happens quite often during the course of experiments, that cells touch each other while migrating up the gradients. In case of any overlap, FITC and DIC images were used in conjunction to manually determine cell boundaries. Subsequently each cell was segmented individually.
Gradient characteristics, namely slope and absolute concentration are lumped together in a single parameter \( \Delta c/c \) while localization of proteins is measured in terms of weighted integral of total normalized fluorescence. The plots of weighted normalized integral against \( \Delta c/c \) show absence of any clear correlation between external gradient conditions and protein localization in both PHAKT-YFP and Actin-YFP cells (Figure 6.3 and Figure 6.4).
Both PHAKT and Actin show comparative orientation distribution that can be fitted under a Gaussian curve (Figure 6.5). The data suggests that internal distribution of Actin and PHAKT may not be influenced by variation of external gradient signals. The result can be of particular significance considering the fact that cells are able to show directional migration even in very shallow gradients. Thus, there may be a different mechanism altogether that governs cell polarization and migration.
6.3.2. Methods: practical challenges and solutions

Unpredictable behavior of cells

HL-60 cells are cancer cell lines. Therefore, sometimes they behave in an unpredictable fashion. The occasional variability was observed primarily in migratory response of cells. Newly thawed cells in the initial stages of subculture showed polarization when stimulated with chemoattractant gradients. But either cells did not migrate at all or migrated to very short distances. Occasionally, problems in cell adhesion were also observed. Even though starting with same seeding density, degree of cell adhesion on the substrate was very low. Cells recovered after a few passages and usually responded in a predictable way in terms of adhesion and migration. During the course of culture, the non-responsive behavior of cells was often observed again.
such cases, cells were consistently subcultured until they recovered. However, if the passage number was too high and cells were unable to recover, then cells were discarded and freshly thawed stock of cells was used to begin with. To maximize the possibility of having good cells to work with and to minimize time delays due to unexpected behavior of cells, they were cultured in three petridishes in parallel, such that one petridish was ready every alternate day for conducting experiments. The erratic occurrences in cell behavior can possibly be reduced, if not eliminated, by carefully determining a number of other factors, including the best time to passage cells, the optimum dilution, and the plating efficiency at various cell densities.

*Transfection efficiency not 100%*

Cells did not exhibit 100% transfection efficiencies. Therefore, all cells were not fluorescent. In order to obtain more fluorescence data from each experiment, initial cell seeding density was increased. The result was an increase in number of fluorescent cells attached to the substrate. However, caution must be taken to not increase cell seeding densities excessively, as it may have some undesirable effects on cell-cell signaling and alter cellular response.

*Low fluorescence intensity of cells*

Another commonly faced issue was low intensity of fluorescence in transfected cells. The level of fluorescence intensity showed some variation from cell to cell. Some of the cells exhibited extremely low intensity levels to begin with. Image acquisition at frequent time intervals also results in quenching of fluorescent dyes, further diminishing the signal intensity. In order to address this challenge, cells were sorted using FACS to enrich cells exhibiting high levels of fluorescence intensity.
Magnification of images

Image acquisition using high magnification objectives 40x and 63x allows us to capture fine details about intracellular phenomena. The disadvantage, of course, is that a higher magnification gives a narrower field of view. Entire channel width cannot be covered in the field of view and that could prove to be of hindrance in quantifying cell response to gradient characteristics. To overcome these limitations and also to maximize the number of cells in the field of view, a compromise was made between choosing appropriate magnification and amount of information content in the image. 0.63x40x objective was chosen to image entire width of the channel and at the same time to capture all relevant and intricate details of transformations taking place during neutrophil chemotaxis.

6.3.3. Advantages and limitations of microfluidic gradient platforms

Microfluidic laminar flow based platforms demonstrated here generate stable chemoattractant gradients. The microenvironments provided to cells replicate \textit{in vivo} conditions, in which cells sense and migrate up the concentration gradients. The platforms allow for high degree of precision and control over gradient characteristics—concentration profile as well as absolute concentration. This makes them suitable for correlating specific cell response with gradient configurations [1,3]. A large number of experiments can be designed simply by changing a few parameters as dimensions of channel, flow rates, concentration of incoming solutions and so on. An active pump-induced laminar flow ensures rapid formation of a temporally stable gradient within a matter of few seconds. This is particularly advantageous in case of fast responding cell types as neutrophils [41-44]. Continuous flow can maintain stable gradients for an
indefinite period of time and thereby makes them suitable for conducting long term experiments as long as cells maintain their viability. Microfluidic assays require less number of cells unlike conventional methods. Not only it extends their application to rare cell types but also allows for testing wide range of conditions with minimum sample requirements [18]. The flowing streams also immediately carry away any secreted cellular signaling factors (autocrine and paracrine) [45], allowing us to study the exclusive effect of chemottractants without any interference. These platforms must therefore be limited to the studies where it is desirable to remove any soluble factors regulating cell-cell communication or where such factors are not of crucial importance. The devices can be easily fabricated in a streamlined process using rapid prototyping methods [46,47]. The simplicity of design and fabrication permits fabrication of multiple units on a single chip, where each unit can be tested for an individual gradient condition. Furthermore, optically transparent nature of devices makes it possibly to directly quantify gradient profiles using fluorimetric dyes [19,39]. It also allows for real time visualization of cells in both optical and fluorescent mode of view as they migrate up the concentration gradients. Not only each cell can be individually tracked, but also changes in cell morphology can be scrutinized with respect to gradient characteristics and time. These devices also allow superimposition of gradients in a controlled manner and can serve as powerful tools to investigate the effects of competing signals on cell behavior. By incorporating on-chip valves and pumps, gradient generation can be easily controlled in a more dynamic fashion [27]. Such a device would permit more flexibility in terms of control over cellular microenvironments.
Being a continuous flow based system, the platform generates a small drag force on cells (<0.02 N/m²) [48]. Weakly adhered cells are detached from the substrates by shear force, thus allowing only well-adhered cells to respond to the gradients. Flow patterns also have an influence on cell trajectories in the direction of flow. However, total cell migration distances remain unaffected [48]. The device must be operated at optimum flow rates to minimize any biased migration pattern of cells. Adequate and consistent care must be taken to acquire the images in a region where gradient is well established to ensure proper interpretation of experimental data. Experiments that involve fixing and staining of cells at an intermediary stage of the experiment would necessitate direct and free access to cells attached on the substrate. This can be accomplished by taking alternative routes to assemble the microfluidic devices in a reversible manner. PDMS and glass substrate can be clamped together using binding clips [49] or reversibly sealed together using aspiration techniques [50]. PDMS can be taken apart at any stage during the course of an experiment and cells adhering on the substrate can be appropriately treated and analyzed.

However, the fast migrating nature of neutrophils and limitations of microscopic imaging techniques poses some inherent restrictions in terms of monitoring certain intracellular processes during chemotaxis. Particularly, acquisition of 3D stack of images is difficult when cell is responding very fast to its microenvironment. Any phenomenon that involves variation in properties across cell height unavoidably suffers from the loss of information content during image capture. Addressing these technical challenges is crucial to enable 3D acquisition of images. Deconvolution techniques can subsequently be applied on the acquired images to restore 3D quantitative information
of the original phenomenon [51]. The resultant enhanced image quality and resolution would make these platforms more viable for highly precise quantitative analysis.

**Further challenges: limitations in absolute time measurements**

Cells attached to the substrate are in a buffer environment before being exposed to gradient conditions. Time $t=0$ should be ideally defined as the time instant at which cells are exposed to the chemoattractant and get stimulated. However, due to time taken in connecting the tubings and establishing the gradient, recording of absolute time point is prone to error. One of the ways to address this limitation is to modify the device by introducing valves [27]. Flow of chemoattractant and buffer solutions can be precisely controlled and exact time points can be recorded. Modification in device design, however, brings an added complexity in terms of fabrication, operation and resource requirements. In experiments, where absolute time measurements are not important, these complexities can be avoided and simpler design should serve the purpose fairly well. But in cases, where precise measurements of cell response in a time-dependent manner are of crucial significance, valve-based designs are recommended.

### 6.4. Conclusions

Microfluidic gradient platforms provide a unique combination of precise control over extracellular microenvironments and ability to visualize intracellular processes during chemotaxis. In this chapter, we demonstrated use of these platforms to investigate protein localization under different external gradient conditions. A new methodology was used to quantify protein localization from acquired fluorescence images. The current data suggests that intracellular spatial localization of PHAKT and Actin may not
be influenced by external gradient conditions. The methodology employed here can be further improved by enhanced data acquisition methods.

6.5. References


Chapter 7 Primary neutrophil chemotaxis in multiple chemoattractants

7.1. Introduction

Neutrophils are specialized immune cells that form the front line of body’s defense system. In order to perform immune functions, neutrophils must efficiently migrate from the vasculature to the site of infection. Recruited neutrophils navigate through a complex array of chemotactic signals, including end target chemoattractants produced at or proximal to the pathogenic source (eg: fMLP and C5a), and intermediary chemoattractants produced by the host (eg: IL-8 and LTB4). To successively find their way through such a complex chemoattractant environment, neutrophils require mechanisms to sense and accurately interpret the signals.

Several hypotheses have been proposed to explain how neutrophils integrate and prioritize the signals [1,2]. Studies have shown that neutrophils prefer to migrate toward fMLP and C5a even in the presence of IL-8 or LTB4, indicating existence of an intracellular signaling hierarchy [3-7]. The preferential migration of cells can be plausibly explained by cross-desensitization of chemokine receptors [7,8] or distinct signaling pathways governing chemotactic response to different chemoattractants [9]. However, our understanding of how neutrophils respond to combinations of intermediary endogenous chemoattractants is still limited.

Foxman et al. [10] explored this behavior using under agarose assays and reported that cells respond to vectorial sum of gradients and prefer to migrate to a distant source.
even in the presence of a local signal. None of the studies have reported existence of any signaling hierarchy between intermediary chemoattractants.

Most of our knowledge of neutrophil chemotaxis phenomena in multiple chemotactics cues has been of qualitative in nature. This is because of the inherent limitations of conventional chemotaxis assays used in such studies [11-13]. In this work, we use microfluidic gradient platforms that can generate well-defined, stable and controllable gradients [14-16]. Real time visualization of cell migration makes these platforms very useful for precise quantitative analysis [17,18]. To investigate neutrophil migration, we expose cells to single and dual gradients of end target and intermediary chemoattractants. We report the first experimental observation of oscillatory behavior of neutrophils in conflicting signals of comparable strength. The oscillatory behavior may be explained by the ability of cells to show directional persistence.

7.2. Materials and methods

7.2.1. Neutrophil isolation

Human whole blood samples were obtained from healthy volunteers by venipuncture into sterile glass tubes containing sodium heparin (Becton, Dickinson and Company). Neutrophils were isolated from blood by density gradient centrifugation method using Lympholyte-poly separation media (Cedarlane Laboratories) that is a mixture of sodium metrizoate and Dextran 500. The entire separation procedure including centrifugation was performed at room temperature (18-22°C). 4 ml of anti-coagulated whole blood was carefully layered over 3.5 ml of the separation media and centrifuged for 35 minutes at 450g. Blood separated out into six distinct bands, namely, plasma, monocytes, isolation media, neutrophils, more isolation media, and the red blood cell
pellet. The band containing neutrophils was carefully collected and washed several times with HBSS (without Calcium and Magnesium, Invitrogen Gibco) by centrifugation (350g for 10 minutes). Cells were then resuspended in HBSS (with 2% HSA)), counted and adjusted to desired concentration. Isolated neutrophils were kept in incubator conditions until use. All experiments were performed within 12 hours of isolation.

7.2.2. Microfluidic device fabrication

The microfluidic gradient device comprises of a poly (dimethylsiloxane) (PDMS) slab embedded with microfluidic channels and glass substrate as its base. The microchannels were fabricated using rapid prototyping and soft lithography as described previously [14,16,19]. Briefly, high resolution printing (5080 dpi) was used to print a photomask with design pattern on a transparency film. The mask was used to fabricate 50 μm high SU-8 photoresist features on a Silicon wafer using photolithography. PDMS molds with embossed channels were fabricated by curing the pre-polymer on Silicon master for 2 hours at 65 °C. The PDMS replica was then peeled off the silicon master. Inlets and outlets for the fluids and cells were created in PDMS using a steel punch. The surface of the PDMS replica and a clean glass coverslip (Fisher Scientific) were treated with air plasma for 90 s (Model PDC-001, Harrick Scientific) and irreversibly bonded together to complete the device assembly. The device was connected to a syringe pump (Harvard Apparatus) with PTFE tubings (Cole-Parmer) and 1ml syringes (BD Biosciences) via 23 G 3/4 size needles (BD Biosciences) in order to deliver fluid and cells to the microchannels. Fibronectin (250 μg/ml) was coated inside the microfluidic device for 1 hour at room temperature before use in each chemotaxis experiment.
7.2.3. Microfluidic gradient generation

The gradient formation was verified by infusing fluorescently labeled solution from one inlet and an unlabeled solution from another inlet of the microfluidic device. Controlled diffusive mixing across the interface of laminar flow streams quickly establishes the gradient. Fluorescent images were acquired at different locations along the channel using FITC filter on Zeiss Axiovert 200M microscope. ImageJ was used to analyze fluorescent intensity profiles across the channel width. The profiles suggest formation of a well-defined, linear and stable solution concentration gradient as demonstrated previously [16].

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7.2.4. Chemotaxis experiments in microfluidic device

Cells were washed and suspended in modified Hanks' balanced salt solution (mHBSS) containing 1% Human Serum Albumin (HSA) and injected in the microfluidic device. The device was kept in the incubator (37°C, 5% CO₂) for 20 min to allow cells to attach to the substrate. Unadhered cells were washed away by gently rinsing the channels with mHBSS. Cells were uniformly distributed across the length and the width
of microfluidic channel before being exposed to any flow. Chemoattractant solutions were infused into the device from separate inlets at the flow rate of 0.02 ml/hr to establish soluble concentration gradients. Differential interference contrast (DIC) images and fluorescent images were captured with a Zeiss 40X NA 1.30 Fluar DIC objective on a Zeiss Axiovert 200M microscope. All images were collected with a cooled charge-coupled device camera (AxioCam MR3, Zeiss).

7.2.5. Quantitative Analysis

ImageJ was used to track migration of individual cells. The acquired time lapse images were calibrated to distance. Cells were tracked using the “manual tracking” plug-in in ImageJ. The tracking data was exported to Microsoft Excel and Matlab for plotting and analysis. Chemotactic indices were used to quantify directional migration of neutrophils. Chemotactic index is the ratio of the displacement of cells in the direction of gradient to the total migration distance. Box and Whisker plots were used to show the spread of the data. In case of competing gradients, the microchannel was divided into three sections along the width for analysis. The trajectories for cells starting from each section were recorded and analyzed.

7.3. Results and discussion

7.3.1. Neutrophil chemotaxis in single gradients

We first examined neutrophil migration in single linear gradients of fMLP, IL-8 and LTB4. A broad range of concentrations for each chemoattractant was tested ranging from 0-10 nM to 0-50 nM. Chemotactic indices were plotted for each chemoattractant. Neutrophils showed positive chemoattractive response for all chemoattractants (Figure 7.1). In the absence of any chemoattractants in control experiments, neutrophils did not
get stimulated and showed no movement or very little movement, if at all, in random direction.

Figure 7.1  Box and whisker plots showing neutrophil chemotactic index in single chemoattractant gradients of fMLP, IL-8 and LTB-4

7.3.2. Neutrophil chemotaxis in dual gradients

Neutrophil chemotaxis in intermediary/end-target gradients

To investigate neutrophil response to multiple chemotactic signals, we performed chemotaxis experiments in dual gradients. The first set of dual gradient chemotaxis experiments comprised of IL-8/fMLP gradients and LTB4/fMLP gradients.
Figure 7.2 (cont. on next page)
Cells were initially uniformly distributed throughout the width of the channel. Trajectories of cells starting their migration in each section of channel revealed cell response to competing signals. Figure 7.2a-c shows cell trajectories in LTB4/fMLP dual gradients. LTB4 concentration gradient was from 0 to 1.5 nM with highest concentration at the top of the microchannel. fMLP concentration gradient was from 0 to 25 nM with highest concentration at the bottom of the microchannel. Cells starting in the top section moved towards the bottom, up the gradient of fMLP. Most of the cells starting in the bottom section started to migrate up the gradient of LTB4 but made a U-turn to migrate up the gradient of fMLP. Cells starting in the middle section showed a mixed response. Some of them migrated straight up the fMLP gradient, while others turned around to eventually migrate along the direction of increasing fMLP concentration. The
observations clearly suggest the role of signaling hierarchy in neutrophil chemotaxis. fMLP always dominates over LTB4. Initial migratory response of cells to LTB4 supports the observations made earlier using under agarose assays that show that cells prefer to migrate toward a distant chemoattractant source even in the presence of a local chemoattractant source. Similar observations were made for IL-8/fMLP dual gradients.

**Desensitization experiments**

Additional experiments were designed to determine if presence of one chemoattractant can make neutrophils loose their sensitivity to the second chemoattractant.

![Figure 7.3](image)

**Figure 7.3** Schematic of concentration profile in desensitization experiments. Solid line indicates concentration gradient of chemoattractant A. Dotted lines indicate uniform base concentration of chemoattractant B.

In these experiments, cells were exposed to the concentration gradient of a chemoattractant superimposed with uniform concentration of a second chemoattractant. The absolute concentration of second chemoattractant was systematically varied in successive experiments as shown in the schematic above (Figure 7.3) to determine
concentration dependent behavior. The migratory response of cells was recorded to investigate desensitization phenomena. Cells were tracked in each chemotaxis experiment and their chemotactic indices were plotted against the absolute concentration of second chemoattractant as shown in Figure 7.4.

Figure 7.4 Chemotactic indices in desensitization experiments performed for (a) IL-8 gradients and (b) LTB4 gradients. In each case, uniform base concentrations of fMLP were varied as 0.1, 1 and 10 nM in successive experiments. Plots show mean and standard deviation for each concentration of fMLP. Chemotactic plots suggest a negative correlation between cell migratory response and fMLP concentration in IL-8 gradients (Pearson correlation = -0.71303; p-value = 1.1406e-10) as well as LTB4 gradients (Pearson correlation = -0.71303; P = 1.1406e-10). Chemotactic index decreased as concentration of base fMLP increased. The results indicate that fMLP suppresses chemotactic response of neutrophils to IL-8 and LTB4 in a concentration-dependent manner. However, the other set of experiments in which cells were exposed to fMLP gradients against a background of IL-8 or LTB4 indicated that cell chemotactic response to fMLP gradient was not affected by presence of IL-8 or LTB4. Neutrophils showed effective chemotaxis up the gradient of fMLP even
when background concentrations of IL-8 and LTB-4 were varied. The observations corroborate the role of intracellular signaling hierarchy in determining direction of migration of neutrophils in competing gradients [9].

Neutrophil chemotaxis in intermediary/intermediary gradients

To understand neutrophil migration in chemotactic signals of comparable strength, we performed experiments in dual gradients of IL-8 and LTB4. In short duration experiments for 20 minutes, cells do not seem to show any net effective directional response, in agreement with earlier neutrophil chemotaxis performed with microfluidic devices [17]. Therefore, long-term experiments were conducted ranging over a period of 80 minutes. Cell trajectories over the entire duration of experiment reveal oscillatory behavior (Figure 7.5). Cells migrate up the gradient of the first chemoattractant and then make a turn to migrate up the second chemoattractant gradient. They continue to oscillate between the competing gradients. The migration of cells to a distant source in the presence of a local source is in agreement with published results [10]. We report the first observation of oscillatory behavior of cells in dual gradients of competing strength. The nature of the platforms makes it possible to conduct long term experiments. Microfluidic gradients can be stably maintained for indefinite period of time and cell migration experiments can be performed as long as cells maintain their viability.
Figure 7.5 Cell trajectories in competing IL-8/LTB4 gradients. Y-displacement of cells from their original position is plotted against time. On y-axis, 0 indicates the center of microfluidic channel and positive displacement is towards the top of channel. IL-8 concentration gradient was from 0-10 nM increasing from bottom to top of the microchannel. LTB4 concentration gradient was from 0-15 nM increasing from top to bottom of the microchannel. Each cell trajectory is marked with a different color.

Several reasons can be given to explain the oscillatory behavior of neutrophils. This may be due to some form of sensory adaptation [10,20]. Neutrophils modulate their sensitivities over time. On sensing a decrease in change of their microenvironments, cells adapt more to the conditions and become less sensitive to that chemoattractant. Rather their sensitivity for distant source becomes stronger and they migrate towards the other source. Recurrence of this phenomenon may cause them to oscillate. However, according to Oelz et al. [21], cells are unable to adjust their sensitivities fast enough as they migrate. Therefore, as they move toward one source, they still maintain their sensitivity to the distant one. Both of these propositions are based on temporal mechanisms for modulating sensitivities. Although it has been shown that cells indeed
are able to change their receptor count, but to what extent that plays a role in determining migration of cells in conflicting signals remains an open question.

Our proposition is based on combination of spatial mechanism governing neutrophil migration and observations of cell behavior in uniform chemoattractant fields. Neutrophils exhibit biphasic dose dependent response characteristics to different chemoattractant concentrations [13]. Cells show optimal sensitivity for each chemoattractant within a certain concentration range. The biphasic dose response curve suggests that for given conditions, when cells approach too close to a source, their sensitivities may exceed the peak threshold, and thus their affinity toward the distance source dominates. In this case, there is no temporal modulation of sensitivities. This idea was used to develop a mathematical model to determine cell behavior in multiple chemoattractants (Yuki Kimura, unpublished results). However, this hypothesis does not provide a complete picture. Cells settle at the mid-point instead of oscillating between the two gradients. Therefore, we propose that directional persistence of cells may play a key role in determining cell response to multiple chemoattractants. Cell persistence, the tendency of cells to continue migrating in a direction, once set in motion, is a common phenomenon observed in uniform chemoattractant fields [1,22-24]. We hypothesize that cells may employ this technique to navigate in presence of multiple chemotactic signals as well. In conjunction with aforementioned spatial mechanism, neutrophils have persistence and that allows them to run too far past the midpoint. Therefore, they constantly try to correct themselves and show oscillatory behavior in competing gradients.
Neutrophil chemotaxis is inherently a spatial and temporal process. Since both phenomena are coupled, it is difficult to isolate the components of the process. Nevertheless, directional persistence allows neutrophils to avoid the equivalent of local maxima in a global optimization problem by allowing for a greater range of exploration. It is quite likely that cells employ persistence mechanism to navigate through cacophony of signals encountered in vivo.

7.4. Conclusions

Microfluidic gradient platforms are powerful tools to study macroscale behavior of neutrophils in precisely controlled gradient environments. Using these platforms, we investigated neutrophil chemotaxis in single and dual (superimposed and competing) chemoattractant gradients. We report the first experimental observation of oscillatory behavior of neutrophils in multiple endogenous chemoattractant gradients. The chemotactic behavior of primary neutrophils suggests the role of directional persistence in guiding cells to their targets. These results provide insight into a key component of the inflammatory response and should facilitate development of novel immunotherapeutic strategies.

7.5. References


Chapter 8 Conclusions and future directions

8.1. Introduction

Microscale platforms are emerging as powerful tools for basic and applied biological research. With major developments in microfabrication techniques in recent years, we have unprecedented ability to engineer cellular microenvironments. These platforms can be used to understand fundamental cellular mechanisms and to control cellular behavior to address specific biological issues. The work presented in the previous chapters demonstrated design, development and application of microscale platforms for human health related issues.

8.2. Topographical control of cell behavior

Tendon damage is a major problem in orthopedic field. Successful tendon regeneration necessitates alignment of tenocytes as well as appropriate arrangement of newly synthesized collagen matrix. In this work, micropatterned substrates were used to provide topographical cues to stimulate aligned growth of tenocytes. These platforms were systematically investigated to study the role of topographical factors on response of tenocytes and their potential in tendon repair. Design rules derived from the in vitro studies can be applied in the design of similar in vivo implants to improve tissue regeneration strategies.

Topographical control of cell behavior will definitely facilitate design and development of tissue engineering approaches for tissue repair like peripheral nerve repair [1], and fabrication of prosthetic devices as in cartilage repair [2]. Success of this approach will be greatly determined by fundamental understanding of cell-substrate
interactions and engineering such interactions to elicit desired cell response. Control over geometric configuration, biocompatibility, porosity, degradation and mechanical strength of scaffolds can significantly influence cell proliferation. Simplicity of design, ease of fabrication, and biocompatibility of tissue engineering guides will also be crucial factors to determine efficacy of scaffolds [2-5].

A combination of various other cues can enhance tissue repair to a great degree. For instance, in case of nerve repair, Schwann cells are shown to serve as biological cues. By pre-seeding the Schwann cells on the microgrooved surfaces of polymer conduits, the performance of these implants can be enhanced greatly [6]. Stem cells, the cells in their most undifferentiated state are also emerging as potential candidates for improving biological adaptation of engineered tissue guides [7]. There are numerous possibilities in exploring surface chemistries that enhance cell attachment and growth in a selective way. Chemical patterning techniques need to be extended to wider range of biomedical relevant biomaterials [8]. We also need to ensure stability of chemical patterns, which are more susceptible to degradation by environmental factors than physical patterns. Mechanisms of cell guidance on chemical patterns have not been well-understood so far and need to be investigated. Dynamic patterning is an interesting option to explore where, surface chemistries can be dynamically varied over time and space and its effect on cellular response can be observed. Synergistic effects of topographical and chemical patterns, especially competing effects of these two have not been explored extensively so far. Future experiments must explore the effects of size, shape, spacing, and pattern design for physical and chemical features on cell response. Three-Dimension tissue constructs must be designed that have desired
mechanical strength and physical and biochemical properties to support cellular growth and proliferation. At nanoscale, chemistry and topography converge [9]. Discovering new biomaterials with physical features that mimic chemical species is an interesting area to investigate. Sizes of biomolecules vary a lot; it is a challenge to find optimum technique for patterning biomolecules. For instance, collagen being a larger size molecule, it will be more feasible to go via microfluidic route rather than microcontact printing. The reduction in feature size seems to be a potential parameter to control the cell behavior. However, it will be too early to project these results for in vivo success considering the enormous complexities involved in in vivo environment.

Different cell lines from different sources may respond in a different way to substrate topography. Fibroblasts from various sources may all not respond in same manner. Some of them may not even align. A critical and exhaustive study where cells from various sources are compared will be highly beneficial. There is a tremendous scope of investigating more deeply the relevance of thermodynamic properties like free energy, chemical potential, etc. to biological phenomenon like contact guidance. It is plausible that the concepts that determine spreading of liquids may also be responsible for cell behavior. There are several other possibilities for combining topographical features with various other factors that can significantly enhance cell behavior, including microgravity [10], electric fields [11], and fluid shear stress due to flow [12].

Seeing in synthesis is the key for future. The future research must aim at finding an underlying common thread among various proposed hypotheses that will help understand the phenomenon of contact guidance. Synergistic application of ideas and
principles from different fields will aid in design and development of novel applications for regenerative medicine and tissue engineering.

8.3. Microfluidic platforms for neutrophil chemotaxis

Microfluidic gradient-generating platforms provide precisely controlled and stable environments for cellular studies. In this work, these platforms were employed to investigate intracellular and extracellular events in neutrophil chemotaxis. The optically transparent nature of platforms allows visualization of fluorescent tagged proteins as they localize during cell migration. A systematic investigation of spatial distribution of key regulatory molecules involved in signaling pathways, in response to controlled external gradient conditions have begin to shed light on intracellular processes involved in neutrophil chemotaxis. These platforms were also used to study macroscale cellular response to multiple chemoattractant signals. Quantitative analysis of migration behavior of neutrophils in response to single and opposing gradients of chemoattractants reveal how neutrophils integrate and prioritize multiple chemotactic cues.

Neutrophil chemotaxis is a highly intricate phenomenon. Current understanding about neutrophil chemotaxis mechanism and signaling pathways is like the tip of an iceberg [13]. Still there is a lot more to learn about how these cells interpret external signals and effectively migrate to their unique targets. Our understanding of the key molecular components involved in feedback loops is still in its infancy. In addition to these, there are several important aspects related to neutrophil chemotaxis that need to be investigated, including mechanisms of adaption [14,15], events governing
development of spatial symmetries of chemotactic signals in the absence of actin polymerization, and mechanisms underlying internal gradient amplification [16].

In addition to lack of complete understanding of mechanisms involved in chemotaxis of neutrophils in the presence of single cue, our knowledge of how neutrophils respond to multiple chemoattractive signals is also very limited. It is not clear how these multiple signals are identified and interpreted by our immune system to effect neutrophil migration to the sites of infection and inflammation. The studies performed in this work have begun to provide insights into mechanisms involved in neutrophil migration in multiple gradients. These studies can be further extended to other chemokines, including TNF-alpha, MIP-2 and other growth factors of interest.

Recent progress in microfabrication techniques and materials science enable us to develop novel and sophisticated microscale systems that can replicate complex cellular microenvironments more accurately. This will allow us to unravel the aspects of neutrophil chemotaxis that cannot be addressed with the existent chemotactic assays.

One of such physiologically relevant situations would be navigation of neutrophils to the target infected tissues through extracellular matrix. During their journey, cells encounter physical obstacles in form of extracellular matrix components, or cells of similar or diverse types. While some of these encounters provide the cells with relevant directional cues by means of chemotactic or adhesion signals [17], other encounters may only pose mechanical barriers to cell migration. It would be interesting to design a device that allows creating an environment where cells encounter obstacles as they migrate up the gradient. In such case, a chemotaxing cell would have to somehow overcome the barrier in order to continue chemotaxis. Such a system would allow us to
investigate how neutrophils respond to mechanical cues and incorporate them with the chemoattractant-sensing signaling machinery.

Neutrophil motility has also been shown to be influenced by substrate topography and surface chemistry of substrates [18]. However, the physical mechanisms governing cell response to such cues still remain to be explored. Furthermore, synergistic and competitive effects of physical and chemical guidance cues can be studied by superimposing topographical features with concentration gradient signals.

While flow-based microfluidic platforms provide precise control over chemical gradient characteristics, the continuous flow introduces some inherent limitations. The flowing streams carry away any autocrine and paracrine signaling factors that play a key role in regulation of biochemical activities. Therefore, it is imperative to develop static gradient-generating platforms, particularly for studies where cell-cell communication is an important factor. A few recent attempts have been made in this direction [19,20]. More sophisticated platforms need to be developed that allow us to perform precise quantitative analysis of interactions between exogenous conditions and endogenous intercellular communication.

Overall, there is a gamut of factors that influence neutrophil behavior in vivo during chemotaxis and determine characteristic cell response to stimuli. These factors include intracellular processes, interactions with other cells, interactions with extracellular matrix, and so on, thereby covering a wide range of local and systemic factors. Future research will aim at replicating in vivo cellular microenvironments on lab scale to understand the individual and coordinated role of such factors during neutrophil chemotaxis.
8.4. Summary

In summary, the work presented here aims at development and application of microscale platforms for biological studies. The platforms provide controlled microenvironments for directed cell growth and migration. Systematic and exhaustive quantitative analyses of cellular response under well-defined conditions make these platforms powerful tools to unravel cellular mechanisms underlying key biological phenomena. The ability to engineer cellular microenvironments to elicit desired cell response is likely have a profound impact on design and development of novel tissue engineering and regenerative medicine strategies.

8.5. References


